Herbivore-induced emissions of methanol and ethylene: volatile signals in the defense response of *Nicotiana attenuata*

Dissertation

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1. Introduction

This introduction provides a general background on plant signaling. Firstly, the adaptive responses of plants, which constitute their phenotypic plasticity, are introduced (1.1. Plants: not quite as passive as they look) and four examples of stress perception through environmental cues are described (1.2. Mindless mastery of stress responses). Secondly, an overview of three different plant species that are extensively studied in the context of hormone signaling is given (1.3. Plant species to study plant signaling) and volatile organic compounds (VOCs) as signaling molecules in plants are described (1.4. Volatile signals in the defense response of *Nicotiana attenuata*).

Detailed introductions of the two volatile compounds methanol (MeOH) and ethylene in the context of *Nicotiana attenuata*'s interaction with *Manduca sexta*, its specialized natural herbivore (*Manuscript I* to *III*), with *Sebacina vermifera*, a growth-promoting endophytic fungus (*Manuscript IV*), and with other plants (*Manuscript V*) are presented at the beginning of the respective manuscript.

1.1. Plants: not quite as passive as they look

Despite their sessile life plants actively regulate growth, development, and physiological processes that allow them to survive a constantly changing environment. Their spatially fixed living requires the ability of terrestrial plants to use solar energy, converted by the photosynthetic machinery, to feed themselves (autotrophy). In contrast to the energy-providing process of photosynthesis, the adjustment of the plant's phenotype to the prevailing environment is an energy-demanding task. Environmental heterogeneity is one of the most important selective forces in nature (Hutchings and Kroon, 1994). Physiological and developmental plasticity, known as phenotypic plasticity, involves the perception, processing, and integration of environmental information by an organism (Novoplansky, 2002). In the regulation of the underlying processes that lead to an adapted phenotype lies an intriguing difference between animals and plants. Plants lack a central nervous system (CNS), which allows for precise signal transduction and rapid responses in animals, and must rely instead on a slow, hormone-based set of feedback loops (Givnish, 2002).

What environmental changes do plants respond to, and how do they recognize such changes? The abiotic and biotic surroundings of a plant provide detectable information (cues) which are then translated into specific responses. In the abiotic environment, salinity, drought, radiation, touch, and bending are factors that are identified by such cues as ionic strength, disruption of membrane integrity, conformational change of specific molecules, and turgor (Ballaré, 1999; Braam, 2005; Bray, 1997). Changes in these plant parameters will elicit a specific response that is propagated within the plant by signal networks. Biotic stress is caused by all kinds of organisms: bacteria, fungi, nematodes, arthropods, reptiles, and mammals, as well as

plants of the same and other species. Typical cues of the living environment are chemical compounds that are specific for the attacker or competitor, such as cell wall fragments of fungi and specific bacterial enzymes (Boller, 1995), inceptins, glucose oxidase (GOX), and fatty acid-amino acid conjugates (FACs) in the oral secretions (OS) of attacking lepidopteran larvae (Alborn *et al.*, 1997; Musser *et al.*, 2002; Schmelz *et al.*, 2006), or volatile compounds emitted by neighboring plants (Farmer, 2001; Gershenzon, 2007). Additionally, non-chemical cues, such as time resolved wounding and changed light ratios, inevitable consequences of chewing insects and neighboring plants, elicit specific responses in the plant (Ballaré, 1999; Mithöfer *et al.*, 2005).

Plants are constantly exposed to several cues, most of which are commonplace, and the responses they elicit are daily routine. The questions arise: when do plants experience stress and when and how do they decide to respond? If the detected parameters indicate an unfavorable state for the plant, e.g. a prolonged encounter of this state would lead to a fitness decline the plant's responses can be called stress responses. A fitness cost can be defined as reduced performance of a plant such as lowered seed set or decreased viability of seedlings in the next generation. The permanent protection against an unfavorable state which is not prevailing would imply fitness costs to the plant if the defense or protection is resource demanding. Examples of costly stress responses are pigments that prevent damage by exposure to UV radiation and defense compounds that deter pathogens and herbivores (Baldwin, 1998; Zavala and Botto, 2002). Phenotypic plasticity, the continuous adaptation to mitigate stress, is a means to increase plant fitness in a changing environment by saving costs of permanent protection when not needed. "Thus, although upon casual observation plants look as though they are not doing very much, within, innumerable pathways are working overtime to keep things as they are, while maintaining a constant state of readiness: not quite as passive as they look." (Kepinski and Leyser, 2003)

1.2. Mindless mastery of stress responses

In addition to physical and chemical signals of the fluctuating environment, the organization of cell position and function in developing organs require complex signal transduction circuits. Phytohormones are important signal compounds that regulate growth and development, integrating information perceived by external cues into the regulatory processes of plants. Common hormone-dependent responses are: stimulated or inhibited growth, induced or suppressed apoptosis, activated or inhibited immune responses, regulated metabolism, and controlled initiation of the reproductive cycle. In some cases, one hormone regulates the biosynthesis of another hormone. Classical phytohormones have been described as plant growth regulators: abscisic acid (ABA), auxin (AUX), cytokinins (CKs), ethylene (ETH), and gibberellins (GA). Plant-environment interactions, specifically plant defenses against herbivores and pathogens, rely in addition on the signaling cascades of jasmonic acid (JA) and salicylic acid

(SA). The rapid development of analytical techniques has identified a multitude of other substances that can be characterized as signal compounds, but are not yet defined as phytohormones. This raises the question, what does a substance need to be a signal? An accepted definition of hormone signaling comprises: 1. synthesis or secretion of the signal, 2. signal transport and perception at the receiver location (as close as another cell compartment or as far as another plant), 3. activation of an enzymatic reaction or a whole process, and 4. metabolism of the signal molecule or at least exclusion from its site of action; such exclusion would enable the response to be terminated. Often several hormones are activated during one adaptive response and the same hormone may be activated by different stresses (Figure 1). How plants perceive stresses and elicit appropriate responses, such as altered metabolism, growth, and development is a major preoccupation of plant researchers. Four regulatory circuits in plants and the adapted phenotypes are presented below.

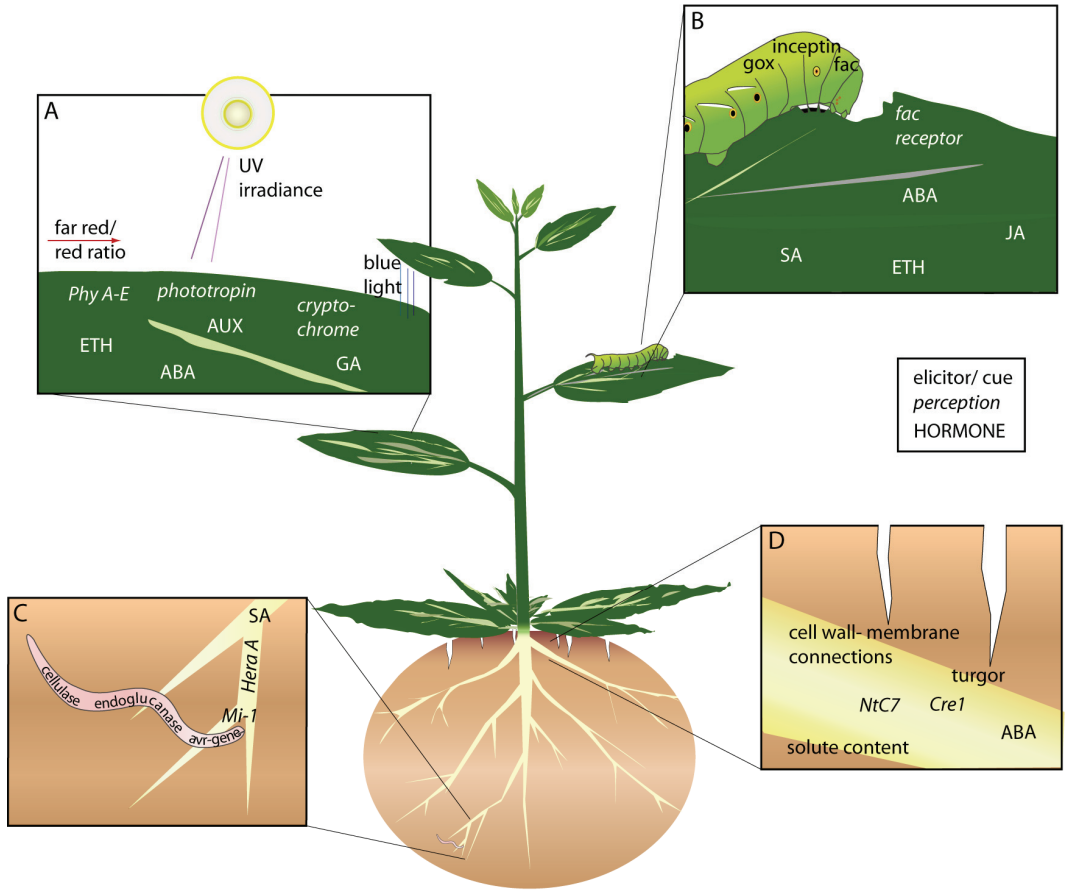
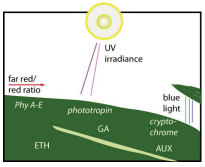
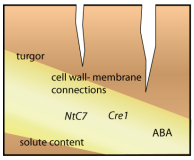


Figure 1. Cues, perception and signal transduction of abiotic and biotic stresses in plants.
A. Ultra violet (UV), far-red (FR), and blue light are perceived irradiances signaling light stress, future competition, and current shading, respectively. Irradiance is perceived by phytochromes (*phy A-E*), phototropins, and cryptochromes that activate reactions through the phytohormones gibberellic acid (GA), auxin (AUX), abscisic acid (ABA), and ethylene (ETH). **B.** Herbivory, e.g. feeding by lepidopteran larvae, is recognized by elicitors in their oral secretions, which include fatty acid-amino acid conjugates (fac), glucose oxidase (gox), and inceptins. Only one receptor-like molecule has been described for perception of the specific fac, volicitin. Above-ground herbivore-

induced defenses are regulated by JA, SA, ETH, and ABA. **C.** Nematodes, below-ground herbivores, are often classified as pathogens and nematode resistance of plants is activated by resistance (r) genes. Surprisingly, so far no cognate nematode effectors corresponding to avirulence genes (avr-genes) of pathogens have been identified. Cell wall fragments or degrading enzymes are further candidates for nematode recognition. Protection against nematodes is regulated by salicylic acid (SA). **D.** Water deficit during drought, salt-stress, and low temperature is perceived through changed ionic strength, cell volume, and turgor differences. Candidate genes of osmosensors are *NtC7* and *Sln1*. The plant's responses are initiated by ABA transport from the root to the shoot.

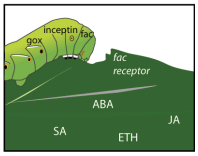


The connection among light signal perception, growth, and developmental pathways allow immobile plants to place structures in an optimized position with respect to water, nutrients, and light. Plants can sense encroaching and future competitors before photosynthetic light levels are reduced via a reduction in the ratio of red (R) to far-red (FR) light reflected by neighboring plants (Ballaré *et al.*, 1990). Upon perceiving a lowered R:FR light ratio, plants react by increasing shoot elongation, hyponasty, and early flowering. This reaction is known as shade avoidance syndrome (SAS) signaled by GA and ETH and potentially AUX (Ballaré *et al.*, 1990; Pierik *et al.*, 2004). Plants perceive R and FR light through a family of five phytochromes (*Phy A* to *E*). Phytochromes exist as two photoconvertible isomers, Pr and Pfr, which absorb maximally in the red and far-red region of the electromagnetic spectrum, respectively (Nagatani, 2004). UV-A and blue light is sensed by phototropins and cryptochromes. While phototropins regulate optimal photosynthesis, including phototropism, chloroplast movement, and stomatal opening through AUX signaling, cryptochromes (*Cry 1* and *2*) regulate de-etiolation, the transition of a dark-grown seedling to a photo-autotrophically competent seedling, photoperiod-dependent flowering induction, and the circadian oscillator via changed sensitivity towards GA and AUX (Chen *et al.*, 2004). As light effects many processes of growth and development, it is not surprising that the phytohormones CK, ABA, and brassinosteroids (BR) are as well directly or indirectly involved in the activated signaling circuits (Zhao *et al.*, 2007).

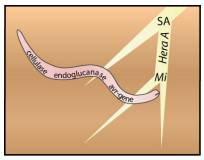


Water deficit in plants occurs when the rate of transpiration exceeds water uptake during drought, salt-stress, and low temperature. On the cellular level, water deficit results in increased solute concentrations, decreased cell volume and loss of the cell wall-membrane integrity, disruption of water potential gradients, and loss of turgor (Bray, 1997). To cope with these stresses, plants produce osmolites for osmotic adjustment, synthesize Na^+/H^+ antiporters for ion sequestration, and protect themselves against oxidative stress with enzymes like glutathione peroxidase, superoxide dismutase, and ascorbate peroxidase. Furthermore, plants adjust their physiological and developmental processes: they

attenuate growth, decrease photosynthesis, and suppress energy-demanding pathways (Bartels and Sunkar, 2005; Zhu, 2001). The protective strategies are activated through perception of changes in turgor, solute content, and the integrity of the plasma membrane-cell wall contacts. Osmosensors have been described for yeast and recently have two candidate plant genes, *NtC7* and *Sln1*, emerged (reviewed in (Bartels and Sunkar, 2005)). In contrast, the downstream signaling pathway is well known. ABA regulates stomatal conductance, leaf epinasty, and root hydraulic conductivity in response to water deficit (Thompson *et al.*, 2007).



A multitude of herbivores feed on above-ground leaf tissue, which plants protect with a bewildering array of defenses: mechanical barriers (e.g. thorns and trichomes), chemical compounds that are toxic to the attacking organism (e.g. alkaloids and glucosinolates), or proteins that reduce the nutritional value of the plant material (e.g. protease inhibitors and polyphenol oxidases). Several defensive traits are inducible and only expressed or increased in response to an attack (Karban and Baldwin, 1997). Herbivore feeding is recognized by herbivore-derived non-enzymatic elicitors, e.g. N-(17-hydroxylinolenoyl)-L-glutamine, a FAC named volicitin, in the OS of *Spodoptera exigua* larvae, which elicits herbivore-induced VOC emissions (Alborn *et al.*, 1997); enzymes, e.g. GOX in the salivary glands of *Helicoverpa zea* larvae, which inhibit the wound-induced accumulation of nicotine in *Nicotiana tabacum* by the producing H_2O_2 at the wound site (Musser *et al.*, 2002); and specific fragments of plant-derived molecules, e.g. inceptins, fragments of ATP synthases, which elicit several defense responses of *Vigna unguiculata* and *Phaseolus vulgaris* (Schmelz *et al.*, 2006). In the plasma membrane of *Zea mays* a putative receptor for volicitin was isolated (Truitt *et al.*, 2004), but so far no other perception molecules for herbivore elicitors have been described. Activation of wound- and herbivore-induced defense responses involves a complex network of plant signaling cascades including peptide signals (e.g. systemin) and phytohormones like SA, ETH, ABA, and JA (Rojo *et al.*, 2003).



Plant parasitic nematodes, e.g. root-knot nematodes (*Meloidogyne* spp.) and cyst nematodes (*Heterodera* and *Globodera* spp.), are pathogens of the kingdom animalia. Nematodes colonize plant roots and rake nutritional resources of their host while they evade or suppress host defenses (Williamson and Kumar, 2006). Symptoms of nematode infestation include root galls, stunted growth, and increased susceptibility to drought stress and pathogen attack (Williamson and Kumar, 2006). Plant resistance to pathogens, including nematodes, is mediated by specialized resistance (r) genes that activate physical and chemical barriers upon recognition of the respective avirulence (avr) gene. The two resistance genes *Mi-1* and *Hera A* are representatives of the NBS-LRR class, referring to the structural similarity to nucleotide-binding site-leucine-rich repeat genes. Valerie

M. Williamson's group made an effort to broaden the knowledge about nematode-plant interactions and the functions of *Mi-1*. *Mi-1* also confers resistance to the potato aphid *Macrosiphum euphorbiae* and the white fly *Bemisia tabaci* (Nombela *et al.*, 2003; Rossi *et al.*, 1998). However, elicitor compounds of nematodes have not yet been described. Root penetration and migration of nematodes in the roots are processes associated with the exudation of cell-wall degrading enzymes like cellulases, β-1,4-endoglucanases, pectate lyases, and expansins (Niblack *et al.*, 2006). These compounds are recognized by plants in their interactions with pathogenic bacteria and fungi (Boller, 1995) and it is likely that cell wall fragments are used for recognition of nematodes. SA has been pinpointed to be involved in the regulation of nematode resistance. Reduced SA concentrations by expression of NahG in roots of tomato plants containing *Mi-1* revealed that SA is required for signaling the defense response (Branch *et al.*, 2004). Although application of JA and methyl jasmonate (MeJA) to tomato roots can alter the performance of cyst nematodes (Cooper *et al.*, 2005), neither nematode-dependent JA regulation nor an increased susceptibility of plants impaired in JA biosynthesis could be demonstrated, questioning JA's function regulating plant responses to nematode infestation.

All interactions described above imply fitness costs for the plant. Nevertheless, mutualistic interactions of plants with their environment, which are highly adaptive, are regulated through comparable signaling networks. Pollinator interactions are facilitated through flower display, which is influenced by ethylene (Patterson and Bleecker, 2004). Predator and parasitoid attraction, ways of defending plants indirectly, are enhanced by the JA-dependent emission of terpenes (Thaler, 1999). Finally, plant growth promotion by fungi and bacteria involves JA signaling (Hause *et al.*, 2002).

1.3. Plant species to study plant signaling

Research on plant stress responses is often motivated by the need to improve crop resistance and decrease the annual yield loss caused by drought and pest outbreaks. Enhanced resistance has long been achieved by elaborate breeding systems but advances in molecular biology allow for the use of transgenic crops to obtain resistant plants in the next or two generations.

Rarely do two species respond in exactly the same way to biotic and abiotic stresses. Most often differences occur at the level of elicitor recognition and during signal transduction (Rojo *et al.*, 2003). From the combined knowledge of several species, general patterns can be identified and might help to understand the evolution of a specific process. The mouse-ear cress (*Arabidopsis thaliana*) has the great advantage of a sequenced genome and a seemingly endless array of available mutants. In addition, our knowledge of signal processes in Arabidopsis is the fastest growing due to its huge research community. Tomato (*Solanum lycopersicon*), an easily transformable crop plant, has a soon to be sequenced genome and a growing community

of researchers working on the system. In contrast, the molecular biologists who study coyote tobacco (*Nicotiana attenuata* Torr. ex Watson, synonymous with *Nicotiana torreyana* Nelson & Macbr.) comprise a relatively small research community. Relatively few gene sequences for this plant are known. The assets of *N. attenuata* are its well-known natural history including herbivore-plant interactions in its natural habitat in combination with an easy and fast transformation system. Furthermore, it colonizes the immediate post-fire environment with nitrogen-rich soils, low interspecific competition, and high intraspecific competition, which resembles the life history of monocultural crops. Knowledge gained in this species is likely to be adoptable for agricultural practice.

Table 1. Comparison of three species used for research on signaling of induced defenses

		mouse-ear cress *Arabidopsis thaliana*	tomato *Solanum lycopersicon*	coyote tobacco *Nicotiana attenuata*
Molecular Biology	genome sequenced	completed	in process	feasible with technical advances
	mutants/ transgenics	many mutants and transgenic plants	mutants and transgenic plants	stable and transient transgenic plants
	transformation procedure	flower dip (fast)	callus re-growth and virus induced	callus re-growth and virus induced
Life History	habitat	mostly solitary plants in disturbed areas and mountain ranges	crop plant; mostly solitaire relatives in mountain ranges	fire chaser; mono-cultural occurrence on nutrient rich soils
	reproduction	selfing and out-crossing	selfing and out-crossing	selfing and out-crossing
Interaction Ecology	natural herbivores	*Trichoplusia ni, Pieris rapae, Plutella xylostella*	*Manduca. quinquemaculata, Bimisia tabaci, Tetranychus urticae*	*Manduca sexta, Epitrix hirtipenis, Tupiocoris notatus*
	defense responses	glucosinolates, PI, PPO, terpenoids,	PI, PPO, tomatin, terpenoids	nicotine, phenolics, and PI, terpenoids
	multitrophic interactions	mycorrhizae and indirect defense	mycorrhizae and indirect defense	mycorrhizae and indirect defense

1.4. Volatile signals in the defense response of *N. attenuata*

As part of a plant's defense response VOCs are emitted; these can transfer information between the plant and other organisms, between plants, and within one plant. Volatiles with known functions, green leaf volatiles (GLVs) and terpenes, attract parasitoids and predators to indirectly defend a plant against the herbivorous hosts of the allured (Kessler and Baldwin, 2001; Turlings *et al.*, 1990), and to deter herbivores in nature (de Moraes *et al.*, 2001; Kessler and Baldwin, 2001). A comparable bouquet could transmit information about the likelihood of herbivory from an attacked plant to its surrounding neighbors: plant-plant communication (Farmer, 2001; Gershenzon, 2007). Candidate signals in plant-plant communication are volatile phytohormones (e.g. ethylene) or volatile derivatives of hormones, such as methylsalicylate (MeSA) and methyljasmonate (MeJA), as these compounds serve as local and systemic signals within a plant and are potent elicitors of plant defense responses (Farmer and Ryan, 1990; Knoester *et al.*, 1998; Shulaev *et al.*, 1997). In addition to the well-characterized emissions of terpenes, GLVs, and phytohormones, other volatiles of unknown function, including MeOH, are emitted in response to herbivore attack (Peñuelas *et al.*, 2005).

The volatile nature of MeOH and ethylene and their potential function as intra- and inter-plant signals motivated the study of their biosynthetic regulation and regulatory ability in the interaction of *N. attenuata* with its natural herbivore *M. sexta* (*Manuscript I* to *III* and *V*). The investigation of herbivore-induced MeOH emissions concentrated on their biosynthetic origin and regulation. After the identification of the elicitor in *M. sexta* OS and the enzymatic pathway that leads to OS-induced MeOH production, the function of MeOH emissions during plant-herbivore interactions was investigated in pharmacological experiments (*Manuscript I*). The biosynthetic pathway of the plant-growth hormone ethylene and its signaling functions in the *N. attenuata*-*M. sexta* and other plant-herbivore interactions -- which were mainly revealed by pharmacological studies -- are well known. The literature about ethylene's role in plant-herbivore interactions was comprehensively reviewed in *Manuscript II*. Using transgenic plants impaired in ethylene biosynthesis and perception, a detailed analysis of ethylene biosynthesis during herbivory was conducted which provided new insights into the regulation of herbivore-induced ethylene emissions (*Manuscript III*). The analysis of ethylene's signaling function using transgenic plants confirmed the existence of at least two ethylene-dependent traits relevant for plant-insect interactions in *N. attenuata*; the herbivore-induced nicotine accumulation and floral longevity (*Manuscript III*). Finally, the crucial role of ethylene in the interaction of *N. attenuata* with the growth promoting fungus, *S. vermifera* was demonstrated (*Manuscript IV*).

The challenges for scientists to identify new volatile signals and to relate new functions to known signals are outlined, and -- with focus on ethylene -- the strategies of plants to integrate a broad range of responses influenced by a single signal without compromising its specificity are discussed.

Manuscript I

Caterpillar-elicited methanol emissions: a new signal in plant-herbivore interactions?

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and Ian T. Baldwin

The Plant Journal (2006) 46: 948-960

Using proton-transfer-reaction mass spectrometry (PTR-MS), we demonstrate that herbivore-induced methanol (MeOH) emissions are elicited by the alkaline pH of *Manduca sexta* larvae oral secretions (OS). OS elicitation increases the transcript accumulation and activity of leaf pectin methylesterases (PMEs), and decreases the degree of pectin methylation. We propose that the released MeOH originates from the activation of PMEs by herbivore attack. Applying MeOH to plants in quantities that mimic the herbivory-elicited release decreases the activity of the potent plant defense proteins trypsin proteinase inhibitors (TPI) and increases the performance of the attacking larvae.

Under the supervision of Ian T. Baldwin I planned and performed all experiments. I measured the herbivore-induced MeOH emission of *Nicotiana attenuata* plants by PTR-MS, isolated and characterized the *NaPME* fragment, and performed the PME activity assay and pectin extractions. The PTR-MS was kindly provided by the group of Robert Schlögl, and Michael Hävecker joined in conducting the MeOH measurements and maintained the PTR-MS.

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Caterpillar-elicited methanol emission: a new signal in plant–herbivore interactions?

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Summary

Plants release into the atmosphere large quantities of volatile organic compounds (VOCs), of which methanol (MeOH), a putative waste product, is the second most abundant. Using online proton-transfer-reaction mass spectrometry (PTR-MS), we demonstrate that when *Manduca sexta* larvae attack *Nicotiana attenuata* plants, the wound-induced release of MeOH dramatically increases. The sustained MeOH emission 24 h after herbivore feeding is already substantially greater than the release of the well-characterized green-leaf VOC *E*-2-hexenal. Herbivore attack and treatment of puncture wounds with larval oral secretions (OS) increased the transcript accumulation and activity of leaf pectin methylesterases (PMEs), and decreased the degree of pectin methylation, as determined by ^{1}H-NMR; therefore, we propose that the released MeOH originates from the activation of PMEs by herbivore attack. The herbivore- and OS-elicited MeOH results not from the activity of previously characterized elicitors in OS but from a pH shift at the wound site when larval OS (pH 8.5–9.5) are introduced into the wounds during feeding. Applying MeOH to plants in quantities that mimic the herbivory-elicited release decreases the activity of the potent plant defense proteins trypsin proteinase inhibitors (TPI), and increases the performance of the attacking larvae. The pH of lepidopteran larvae regurgitants is commonly very high, and the MeOH released during feeding that is elicited by the pH change at the wound site functions as a quantitative signal that influences the outcome of the plant–herbivore interaction.

Keywords: *Nicotiana attenuata*, plant–herbivore interactions, methanol, pectin methylesterase, VOCs, proton-transfer-reaction mass spectrometry.

Introduction

Plants release into the atmosphere considerable quantities of low-molecular-mass hydrocarbons, of which methanol (MeOH) and isoprene are quantitatively the most important after CO_2 (Sharkey, 1996). Most plants release MeOH, with an estimated annual flux from vegetation >100 Tg (Guenther *et al.*, 1995). Wounding increases MeOH emissions, which are commonly at their highest levels during early stages of leaf development; these emissions are thought to be a biochemical waste product that exits the plant through the stomata (Nemecek-Marshall *et al.*, 1995). While the MeOH released from plants can arise from several biochemical sources, the enzymatic demethylation of pectin by pectin methylesterase (PME) is likely to be quantitatively the most important. Pectin polymers account for 35% of the cell wall mass of dicotyledonous plants, and consist of α-D-galacturonic acid units with variable amounts of methyl esters. The esterification of pectin is thought to increase the fluidity of the cell wall. Demethylation of pectin during leaf maturation produces free carboxylated side chains that can be cross-linked by calcium ions, making the cell wall more rigid (Galbally and Kirstine, 2002). Other potential sources of MeOH are the ubiquitous protein repair system, protein L-isoaspartate (D-aspartate) *O*-methyltransferase (PIMT), and lignin degradation, an enzymatic demethylation process performed by a variety of wood-rotting fungi and bacteria (Fall and Benson, 1996). In comparison with other volatile organic compounds (VOCs) released by plants into the atmosphere, the atmospheric half-life (16 days) of MeOH released from plant surfaces is relatively long (Singh *et al.*, 1995), which allows for long-range transport and

participation in the photochemistry of the troposphere as well as in various plant–environment interactions.

The *in planta* function of MeOH is still a matter of debate. Studies have reported that when C3 plants grown in arid environments are sprayed with 10–50% MeOH solutions, growth and yield increase. The proposed mechanism for the increase in growth is the increased carbon fixation resulting from detoxification by the photorespiratory and other metabolic pathways (Nonomura and Benson, 1992). Using ^{13}C-NMR, MeOH was found to be metabolized to serine, methionine and phosphatidylcholine in sycamore cells (Gout *et al.*, 2000), and its growth-promoting activity in microalgae was found to be light-dependent and perhaps the result of increased concentrations of CO_2, the end-product of MeOH metabolism (Theodoridou *et al.*, 2002). However, conflicting effects of MeOH on growth have also been reported (Hartz *et al.*, 1994; Hemming *et al.*, 1995). Furthermore, methylotrophic bacteria, colonizing the leaf surface as non-pathogenic microbes, metabolize MeOH and may influence plant growth and development (Holland, 1997).

In contrast to atmospheric chemists, who have focused on the quantitatively important VOCs, MeOH and isoprene, chemical ecologists have focused on less abundant plant VOCs, such as terpenoid and green-leaf VOCs (GLVs); these play an important role in defense signaling in herbivore-attacked plants. These compounds function as indirect defenses by attracting the natural enemies of the herbivores (Turlings *et al.*, 1990), as direct defenses by repelling the herbivores (DeMoraes *et al.*, 2001; Kessler and Baldwin, 2001), and possibly as signals mediating between-plant interactions (Dicke and Bruin, 2001; Farmer, 2001). These herbivore-elicited VOCs, which can be highly species- and interaction-specific, allow parasitic wasps to find their particular hosts in laboratory studies (DeMoraes *et al.*, 1998). In field experiments, however, individual components of the herbivore-induced VOC blend are sufficient to attract generalist predators (Kessler and Baldwin, 2001).

Many of these VOCs are elicited not by mechanical damage but when herbivore-specific elicitors from oral secretions (OS) or oviposition fluids are introduced into wounds during a plant–herbivore interaction. Elicitors in OS and oviposition fluids include β-glucosidase, glucose oxidase, fatty acid–amino acid conjugates (FACs) and α,ω-diols (Alborn *et al.*, 1997; Doss *et al.*, 2000; Eichenseer *et al.*, 1999; Halitschke *et al.*, 2001; Mattiacci *et al.*, 1995). The *Manduca sexta–Nicotiana attenuata* system has been intensively studied in this regard, and most of the herbivore-specific responses that are mediated by the plant's jasmonate (JA) cascade are elicited by FACs found in the OS of *Manduca* larvae (Halitschke *et al.*, 2001, 2003b).

Chemical ecologists and atmospheric chemists have used different analytical instrumentation to analyze plant VOCs. Coupled gas chromatography (GC) – electron ionizations (EI) mass spectrometry (MS) is particularly useful for analyzing most GLVs and terpenoid VOCs but not for detecting most oxygenated VOCs, such as MeOH, which requires pre-concentration and readily partitions with water in the sampling devices (de Gouw *et al.*, 2000). Furthermore, a common procedure for headspace analysis is the pre-concentration of emitted VOCs on adsorbents and the subsequent elution with solvents that usually have a boiling point comparable with that of MeOH and thus co-elute with MeOH from the GC column. Moreover, the identical molecular masses of O_2 and MeOH ($m/z = 32$) complicate detection under traditional EI conditions. Proton-transfer-reaction mass spectrometry (PTR-MS), a procedure popularized by atmospheric chemists to detect VOCs with proton affinities >166.5 kcal mol^{-1} (proton affinity of H_2O) at sub-ppb (parts per billion) levels, is ideally suited for detecting trace gas emissions online. This new technology, which allows real-time analysis without pre-concentration and chromatography, has recently been used by ecologists to analyze VOCs released from Arabidopsis roots (Steeghs *et al.*, 2004) and *Succisa pratensis* leaves (Peñuelas *et al.*, 2005).

Using PTR-MS to characterize VOC emissions from *N. attenuata* plants attacked by *M. sexta* larvae, we discovered that MeOH emissions are dramatically amplified when plants are under attack, probably as a result of the enzymatic demethylation of pectin. In addition, we have determined that it is the high pH of the larval OS that is responsible for elicitation of the MeOH burst. To examine its function, MeOH was applied to plants in quantities that mimicked the herbivore-elicited release. The ability of MeOH-treated plants to elicit an important herbivore resistance trait, trypsin proteinase inhibitor (TPI) activity, was impaired; moreover, larvae fed MeOH-treated plants grew significantly faster than those fed water-treated control plants. These results suggest that the herbivore-elicited MeOH release should be considered a signal in plant–herbivore interactions, and that the presumption that MeOH is a waste product should be reconsidered.

Results

Plant-derived MeOH released during caterpillar feeding exceeds its emission from control plants

PTR-MS was used to monitor online trace gas emissions of *N. attenuata* plants under herbivore attack and those of control plants. The sampling device enabled us to track the concentrations of several organic components in the headspace of six individual plants (Figure 1). The headspace was sampled sequentially at 10 min intervals to determine the concentration of the following product ion masses (m/z): 31 (1%, MeOH), 33 (99%, MeOH), 37 (water clusters), 57 (23%, *E*-2-hexenal), 81 (58%, *Z*-3-hexenal; 67%, monoterpenes), 83 (80%, *E*-2-hexenol, *Z*-3-hexenol and *E*-2-hexenyl acetate),

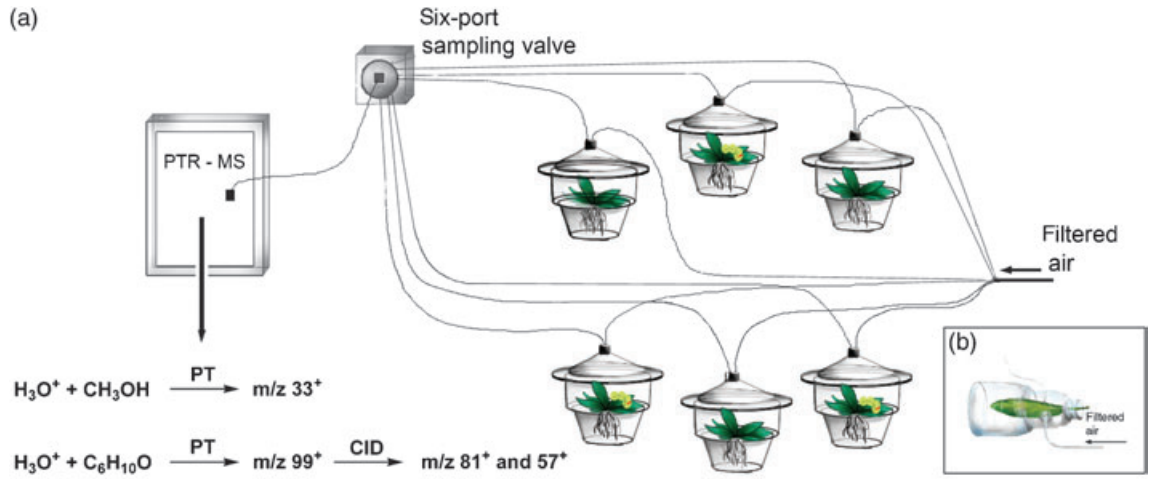

Figure 1. Experimental set-up used for measurements of MeOH emissions by PTR-MS.
(a) Schematic of whole-plant volatile collection. Compressed air was filtered by a zero-air generator and passed through six 1 l desiccators, each housing a hydroponically grown *Nicotiana attenuata* plant. Half of the plants were attacked by five freshly eclosed *M. sexta* larvae; the other three plants were not attacked. The air from these six individual plant chambers was sequentially analyzed (as controlled by a six-port sampling valve) for 10 min from each desiccator over 40 h (see Figure S1 for complete time course). We measured the concentration of the following product ion masses representative for the compound given in parentheses with the percentages of the fragment ion abundance: 31 (1%, MeOH), 33 (99%, MeOH), 37 (water clusters), 57 (23%, *E*-2-hexenal), 81 (58%, *Z*-3-hexenal and 67% monoterpenes), 83 (80%, *E*-2-hexenol, *Z*-3-hexenol and *E*-2-hexenyl acetate) and 137 (33%, monoterpenes); CID, collision-induced dissociation; PT, proton transfer. (b) For single-leaf measurements, whole leaves were inserted into a 275 ml flow-through chamber supplied with filtered air at a rate of 275 ml min^{-1}, and a fraction of that air (15 ml min^{-1}) was analyzed by PTR-MS.

and 137 (33%, monoterpenes; de Gouw *et al.*, 2000; Holzinger *et al.*, 2000).

MeOH had an emission pattern comparable to that of GLVs, as represented by the concentrations of *m/z* 33 for MeOH and *m/z* 57 for *E*-2-hexenal (Figure 2). *N. attenuata* plants attacked by *M. sexta* larvae released

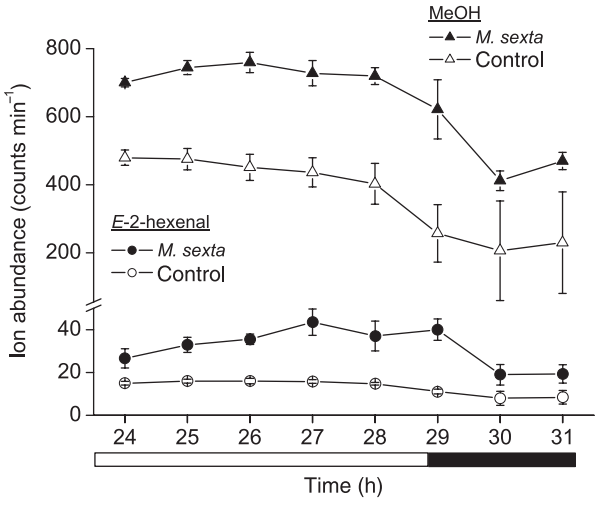

Figure 2. MeOH and *E*-2-hexenal released from *N. attenuata* plants when attacked by *M. sexta* larvae.
Mean ion abundance of *m/z* 33 (MeOH, triangles) and *m/z* 57 (*E*-2-hexenal, circles) per min ± SE sampled in the headspace of non-attacked plants (open symbols) and plants that were under continuous attack by five neonate *M. sexta* larvae (filled symbols). Data points represent the integrated peak area of 10 min intervals. Data are presented for an 8 h time period 24 h after the start of larval attack. Open and black bars designate light and dark periods, respectively.

700.05 ± 12.58 counts min^{-1} (*m/z* 33, MeOH) and 27.68 ± 4.63 counts min^{-1} (*m/z* 57, *E*-2-hexenal) 24 h after the initiation of herbivore feeding, which significantly exceeded the release from control plants (479.54 ± 22.54 counts min^{-1}, MeOH, *t*-test, $P < 0.01$ and 15.59 ± 0.09 counts min^{-1}, *E*-2-hexenal, *t*-test, $P = 0.05$; Figure 2). MeOH is not fragmented during protonation with H_3O^+ ions, and thus *m/z* 33 represents >99% of the ionized MeOH, whereas *m/z* 57 represents one-quarter of the fragment ions of E-2-hexenal produced by ionization with H_3O^+ (product ion masses: 70% *m/z* 99, 23% *m/z* 57, 6% *m/z* 81). The overall VOC release decreased during the dark phase (Figure 2 and Figure S1). The smaller differences between control and caterpillar-attacked plants may be due to the reduced feeding activity of larvae during the night, as indicated by the reduced *m/z* 57 levels, which correlate with the release of GLVs from fresh wound sites on the plant. DeMoraes *et al.* (2001) reported increased GLV emissions of plants attacked by the nocturnal *Heliothis virescens* larvae at night, but several herbivore-induced volatiles have a diurnal emission pattern with higher emissions during the day. Alternatively, the reduced rate of emission might originate from decreased stomatal conductance (Nemecek-Marshall *et al.*, 1995). Overall, herbivore attack increased the release of MeOH by 30%.

A replicate experiment, in which plants were weighed after the analysis, revealed emissions of 1.7 ± 0.17 μmol min^{-1} g^{-1} fresh mass [(FM); 811.36 ± 139.4 counts min^{-1}] 24 h after the onset of caterpillar attack, compared with 1.35 ± 0.12 μmol min^{-1} g^{-1} FM (619.53 ± 53.17 counts

min[1]) from control plants (Figure S1). Thereafter, MeOH emissions differed between attacked and control plants by about 30%. In addition, the well-documented increase in MeOH emissions in the morning was found to be light-dependent, rather than the result of a circadian response; MeOH emissions did not increase in the morning in plants grown under an extended light phase (Figure S1).

Herbivore-induced MeOH emission is triggered by a pH shift at the wound site

To determine whether wounding alone could account for the increase in MeOH emissions from caterpillar-attacked plants, we wounded single leaves with a fabric pattern wheel, which produced three rows of puncture wounds on each side of the midvein, and measured the emission from the wounded leaf over a period of 5–10 min. Thereafter, we treated the wounds with water or OS from *M. sexta* larvae and measured the emission for an additional 5–10 min (Figure 3, inset). When wounds were treated with water, MeOH emissions barely increased by 16.05 ± 6.67–116.03 ± 17.03 μmol min^{-1} g^{-1}, while the addition of OS to wounds almost doubled the release rate (Table 1, *t*-test, $P < 0.001$).

We hypothesized that the FAC-based elicitors of the *Manduca* OS, which elicit the release of terpenoid VOCs, would also elicit the release of MeOH. Interestingly, this was not the case. A mixture of two synthetic FACs, the most abundant in *M. sexta* OS (Halitschke *et al.*, 2001) and sufficient to induce several herbivore-specific changes and most of the JA-related changes, was not active. Yet when all FACs were removed from OS by ion-exchange chromatography, the FAC-free OS were also not active (Table 1, *t*-test, $P = 0.955$), suggesting that either less abundant FACs are responsible for the MeOH release or that passing OS through an ion-exchange column removed the elicitors in addition to the FACs. We tested an additional protein known to occur in OS, glucose oxidase (Eichenseer *et al.*, 1999), and found it to be inactive (Table 1, *t*-test, $P = 0.193$). Elicitors of pathogen responses, chitosan, polygalacturonic acid (PGA) and hydrogen peroxide, did not actively induce the MeOH release (Table 1, *t*-test, $P > 0.256$). We boiled OS and treated OS with proteinase K to denature protein-based elicitors, and found that both types of denatured OS retained their activity (*t*-test, $P < 0.001$).

Finally, to determine whether plant hormones were involved in the MeOH release, we treated wounds with SA, JA, and ethylene and found that none elicited MeOH emissions (Table 1, *t*-test, $P > 0.089$). The low total emission rate after JA application was not due to an inhibitory effect, as is apparent when the induced release rate after JA treatment is compared with the water-induced MeOH emissions (Table 1, *t*-test, $P > 0.262$). Furthermore, we measured MeOH emissions after applying abscisic acid (ABA), which induces PIMT activity, but ABA did not increase the release of MeOH. Induced emissions were comparable to those of water-treated wounds and were significantly less than the OS-induced MeOH emission (*t*-test, $P < 0.05$).

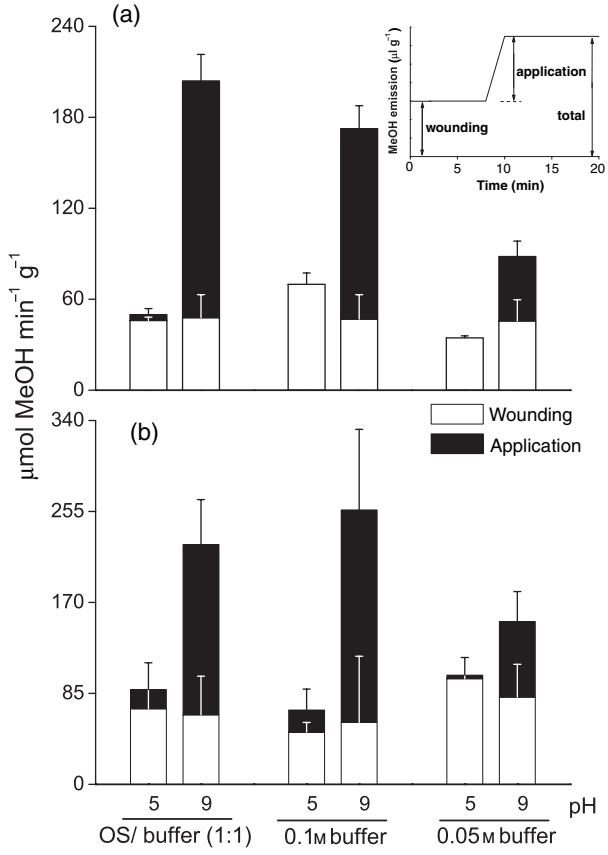

Figure 3. Wound-induced MeOH emission is amplified by a pH change that results from the introduction of herbivore oral secretions (OS) into the wounds during feeding.
Mean μmol MeOH min^{-1} g^{-1} FM ± SE released from single *N. attenuata* leaves after standardized puncture wounding and application to the wounds of 20 μl of (a) *M. sexta* OS diluted 1:1 with 0.1 M phosphate buffer with pH 5 and 9, 0.1 M phosphate buffer with pH 5 and 9, and 0.05 M phosphate buffer with pH 5 and 9, and (b) *M. sexta* OS diluted 1:1 with 0.1 M MES (pH 5.5) and 0.1 M Tris (pH 9), 0.1 M MES (pH 5.5) and 0.1 M Tris (pH 9), and 0.05 M MES (pH 5.5) and 0.05 M Tris (pH 9). Wound-induced MeOH emission (white bars) and MeOH emission elicited after the test solutions were applied to the puncture wounds (black bars) are presented. Inset: Schematic illustrating the time course of the measurements for wound, induced and total emission.

Analyzing these results, we realized that the eliciting activity of the test solutions positively correlated with their pH. Lepidopteran larvae are known to have extraordinarily alkaline midgut fluids (Berenbaum, 1980). Application of different buffers with different pHs to puncture wounds revealed the highest elicitation of MeOH emissions at pH 8 and pH 9 (Figure S2), values equivalent to the pH of *M. sexta* OS reared on an *N. attenuata* leaf diet (pH 9.3). Additionally, we wished to determine whether manipulation of the pH and the molarity of different solutions influenced MeOH

Table 1 Induced MeOH emissions by different insect-, pathogen- and plant-derived elicitors

Test solution	Total emission (μmol min^{-1} g^{-1} FM) ± SE	Induced emission (μmol min^{-1} g^{-1} FM) ± SE
Water control	116.03 ± 17.03	16.05 ± 6.67
M. sexta OS	218.98 ± 25.68**	173.56 ± 19.5*
FACs	80.48 ± 4.44	15.31 ± 13.83
Ion-exchanged OS	114.3 ± 22.96	42.71 ± 13.83
Jasmonic acid	62.46 ± 6.17	27.9 ± 4.44
Salicylic acid	67.89 ± 11.6	21.48 ± 9.38
Triton	74.8 ± 9.13	20.24 ± 13.08
Ethylene	145.41 ± 20.98	21.73 ± 29.63
H_2O_2	94.8 ± 8.15	26.08 ± 4.94
Chitosan	96.28 ± 17.03	17.03 ± 6.17
PGA	87.89 ± 4.44	6.17 ± 10.62
Glucose oxidase	82.95 ± 6.17	25.43 ± 9.38
Boiled OS	338.72 ± 39.01**	250.83 ± 48.14**
Enzyme-free OS	281.44 ± 27.4**	195.03 ± 21.73**

Single leaves of *N. attenuata* plants were wounded with a fabric pattern wheel and their MeOH emission was monitored for a 5–10 min period. After the application of 20 μl of elicitor-containing solutions to the puncture wounds, the emission was measured for an additional 5–10 min. Wound-induced MeOH emission was measured by PTR-MS immediately after wounding and subtracted from the emission measured after application of the elicitor solution (total emissions) to determine the induced emission (see Figure 3, inset). Known herbivore-derived elicitors [oral secretions (OS), FACs and glucose oxidase], plant hormones (jasmonic acid, salicylic acid and ethylene) and pathogenic elicitors (H_2O_2, chitosan and PGA) were tested ($n = 3$, except for *M. sexta* OS, $n = 6$). Asterisks represent levels of significant differences between the elicitors and the water-treated control (*t*-test, $P < 0.01$ and $P < 0.001$).

emissions. Therefore, we diluted the 0.1 M buffers with water to decrease the molarity and with OS to change the pH. Manipulation of the molarity significantly decreased MeOH emissions only for the phosphate buffers (two-way ANOVA $F_{2,13} = 31.016$, $P < 0.001$). The same trend was apparent when the biological buffer was mixed with water (two-way ANOVA $F_{2,13} = 0.049$, $P = 0.9519$; Figure 3). This suggests that the molarity of the buffer and its capacity to maintain a certain pH at the wound site are important for elicitation. The application of any solution with a pH of five did not elicit MeOH emissions regardless of the mixture (two-way ANOVA; phosphate buffer: $F_{1,13} = 189.946$, $P < 0.001$; biological buffer: $F_{1,13} = 20.47$, $P < 0.001$). Manipulation of the pH of *M. sexta* OS with both buffer systems demonstrated that pH elicited the release of MeOH (Figure 3).

The enzymatic demethylation of pectin by pectin methylesterase (PME) is the source of OS-induced MeOH emission

Two sources of MeOH emission from plants have been suggested. Of these, the protein repair system, protein L-isoaspartate (D-aspartate) *O*-methyltransferase (PIMT), is unlikely to contribute significantly to differences in MeOH emissions. PIMT-induced emissions by leaves are typically in the range of pmol min^{-1} g^{-1} FM (Thapar *et al.*, 2001). Such differences would not be detectable in the μmol min^{-1} g^{-1} FM releases that are elicited by caterpillar attack, and were measured in the elicitor experiments. ABA, dehydration and salt stress are known to increase methyltransferase mRNA and activity, but in our experiments applying ABA to wounded leaves did not increase MeOH emissions. Hence, we determined whether the enzymatic demethylation of the variously methylated pectin polymers ubiquitously found in cell walls by PME could be the source of caterpillar-induced MeOH emission (Figure 4a).

We used ^{1}H-NMR to rapidly measure free and bound MeOH both before and after saponification of pectin extracts to estimate the level of their *O*-methyl residues (Figure S3). As the release of MeOH after wounding and OS application reaches a maximum between 15 and 30 min as determined by PTR-MS (Figure S4), we extracted leaf pectin after 30 min and measured the amount of free MeOH before and after saponification (Table 2). Alkaline conditions cleave the *O*-methyl linkages of the galacturonic acid residues, allowing detection and integration of the H-4 signal of the newly unesterified pectin.

The increase of free MeOH released by saponification of the pectin extracts of control leaf samples (9.6-fold) was significantly higher than the increase of free MeOH from pectin extracts of *M. sexta* OS-treated wounded leaves (5.0-fold; *t*-test, $P < 0.05$, log-transformed). The increase in free MeOH released by saponification of the pectin extracts of wounded leaves differed substantially from the increase measured in control samples (6.9-fold; *t*-test, $P = 0.2964$, log-transformed) but exceeded the increase of OS-treated samples (*t*-test, $P = 0.4052$, log-transformed; Table 2). The larger increase of free MeOH after saponification reflects the higher degree of methylation of samples from wounded and water-treated plants in comparison to wounded and OS-treated plants, which is consistent with the hypothesis that demethylation of pectin is the source of released MeOH.

The observed differences in demethylation may be due to enzymatic activity or direct chemical demethylation of pectin. The first evidence that MeOH emission was caused by enzymatic demethylation of pectin rather than a chemical process alone came from changes in the transcript accumulation of genes encoding for PMEs. Transcriptional analyses using the TIGR 10 k cDNA potato microarray, which compared the transcriptome of *M. sexta*-infested *N. attenuata* plants with control plants, revealed that PME transcripts accumulated in response to herbivory (Schmidt *et al.*, 2005). The *Solanum tuberosum* PME (BQ505674) with high similarity to the *N. attenuata* PME (*NaPME*; Figure S6, accession number DQ115979) showed the largest up-

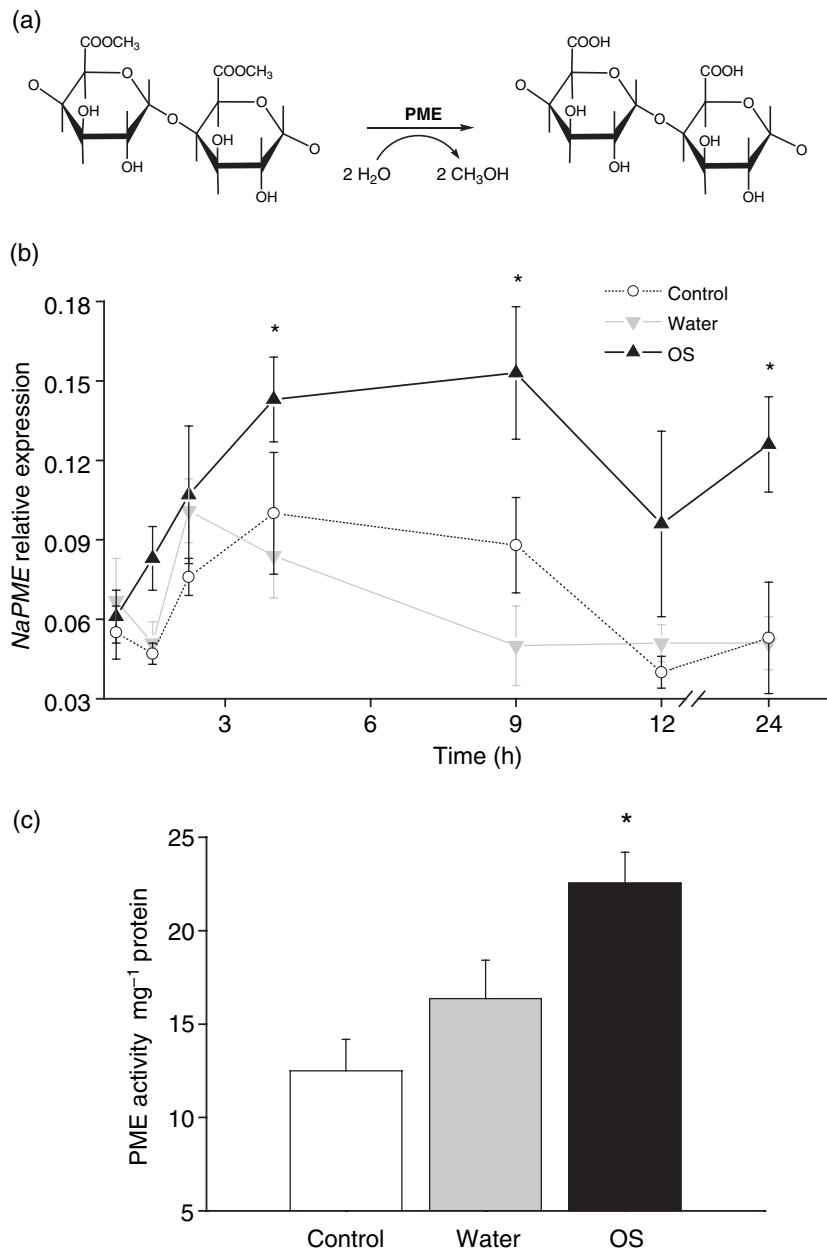

Figure 4. The initial OS-induced MeOH release is associated with a rapid increase in enzymatic demethylation of pectin by PME, and a delayed increase in PME transcripts.
(a) Pectin polymers consist of α-D-galacturonic acid units (dimer shown) with varying degrees of methylation. Demethylation of the pectin polymers by pectin methylesterase (PME) releases one molecule of MeOH per α-D-galacturonic acid unit. MeOH is released into the apoplast and finally emitted through the stomata.
(b) Relative expression ratio ±SE in arbitrary units of *NaPME* transcripts as analyzed by RT-PCR. cDNA was synthesized from total RNA isolated from five replicate *N. attenuata* plants harvested at the indicated time points after single leaves were wounded (time: 0 h), and the puncture wounds were immediately treated with water (grey triangles) or 1:1 diluted *M. sexta* OS (black triangles) and from control plants (circles). Asterisks represent significant differences between *NaPME* expression in OS- and water-treated plants (*t*-test, $P < 0.05$).
(c) PME activity units mg^{-1} protein ±SE of *N. attenuata* leaves 30 min after leaves were wounded and the puncture wounds treated either with 20 μl water or 1:1 diluted *M. sexta* OS, and PME activity of untreated control leaves. Asterisks represent significant differences from the control (ANOVA, $F_{2,15} = 7.971$, Bonferroni ≤0.01).

regulation (ER = 2.59) of PMEs available on the microarray. Altogether four of the five PME-like ESTs regulated in *Solanum nigrum* or *N. attenuata* were up-regulated after *M. sexta* attack in wild tobacco (BQ117605 ER = 1.59, BQ518540 ER = 1.94, BQ512456 ER = 1.96) and for one no data was produced (BQ515829); none were down-regulated. Real-time PCR analysis of *NaPME* revealed two expression patterns: diurnal, which roughly tracks the pattern of higher MeOH emission during the day and reduced emissions during the night (Figure 2, Figures S1 and S5), and OS-elicited (Figure 4b). *NaPME* transcripts are more abundant when puncture wounds are treated with *M. sexta* OS than with water. The significant differences observed 4 h after the treatment are maintained for at least 24 h (Figure 4b, *t*-test, $P < 0.05$).

This increase in mRNA is too slow to account for the wound-induced MeOH release after 15 min but may contribute to the sustained MeOH release observed in *M. sexta*-attacked plants. To determine whether OS are able to activate PMEs, we measured PME activity with a gel diffusion assay 30 min after treating wounds with either water or *M. sexta* OS. We found that OS elicit significant increases in PME activity compared to untreated control leaves (*t*-test, $P \leq 0.05$); wounding alone increased PME activity moderately (Figure 4c). The observation that OS induced an increase in PME activity that was not preceded

Table 2 Inferred demethylation of pectin by ^{1}H-NMR

Elicitor	Free MeOH before saponification (μmol)	Free MeOH after saponification (μmol)	Fold increase due to saponification
Control	13.23 ± 0.62	125.6 ± 22.44	9.68 ± 2.13
Water	12.82 ± 0.67	88.9 ± 22.91	6.92 ± 1.81
M. sexta OS	12.3 ± 0.73	60.84 ± 3.59*	5.03 ± 0.47*

Free MeOH in μmol ±SE normalized to tetramethylsilane (TMS) before and after saponification with 1 M NaOD detected by ^{1}H-NMR in pectin extracts of *N. attenuata* leaves (Figure S3). MeOH levels in pectin extracts of control leaves and of wounded leaves that have been treated with 20 μl of either *M. sexta* OS (1:1 diluted with water) or water 30 min before harvest, and the mean (±SE) fold increase of MeOH after saponification were measured. Asterisks represent significant differences between the elicitor and the control (*t*-test, $P < 0.05$, log-transformed).

by a transcript accumulation is consistent with the direct activation of PME by OS.

Methanol treatment increases M. sexta *larvae performance on* N. attenuata *plants*

As the emission of both herbivore-induced terpenoids and GLVs is known to influence herbivore–plant interaction by attracting parasitic wasps or predators and by functioning as a feeding stimulant, respectively, we asked whether the herbivore-induced MeOH release influenced caterpillar growth, thus altering the outcome of the herbivore–plant interaction. We sprayed plants that had an approximate shoot weight of 7 g with a 5% MeOH solution to deliver 100 μl of MeOH at each spraying, the amount released in 6 h by an attacked plant weighing 4.5 g. Plants were sprayed three times per day over a 2-day period. *M. sexta* larvae feeding on MeOH-sprayed plants gained twice as much body mass after 9 days as larvae that were fed water-sprayed control plants (Figure 5, ANOVA, $F_{1,10} = 8.262$, $P < 0.05$). These results demonstrate that larvae perform equally well when fed MeOH-sprayed plants as when fed plants that have been transformed or naturally silenced in the expression of potent anti-herbivore defense proteins, TPIs (Glawe *et al.*, 2003; Zavala *et al.*, 2004). To explore this potential association, we measured TPI activity levels and found them to be 30% lower in both source and sink leaves of MeOH-sprayed plants than in water-sprayed controls (Figure 5, two-way ANOVA, $F_{1,16} = 4.355$, $P = 0.05$).

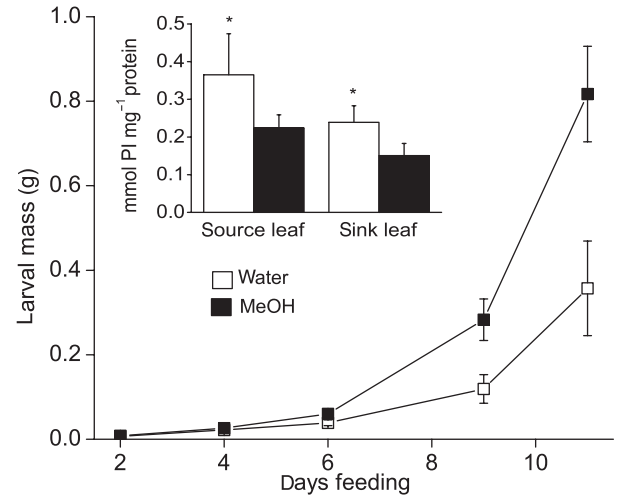

Figure 5. *M. sexta* larval performance on *N. attenuata* plants after MeOH treatment.
Mean larval mass ± SE of *M. sexta* larvae feeding on individual *N. attenuata* plants that had been sprayed three times with 100 μl MeOH delivered in a 5% aqueous solution (filled symbols, $n = 7$) and 100% water (open symbols, $n = 6$) over 2 days before the neonate larvae were placed on the youngest fully expanded leaf. Inset: Mean ± SE trypsin proteinase inhibitor (TPI) activity of leaves at the onset of herbivore attack. Asterisks represent significant differences between MeOH- and water-treated plants (two-way ANOVA, $F_{1,15} = 4.355$, $P \leq 0.05$, log-transformed).

Discussion

The large amount of MeOH released from vegetation has long been assumed to be a metabolic waste product. We report three discoveries that should prompt plant biologists to reconsider this assumption: (1) herbivore attack increases an already large MeOH release; (2) lepidopteran larval OS are the elicitor regulating PME activity and the demethylation of pectin, and the pH of OS regulates the release of MeOH; and (3) the MeOH emission alters the outcome of the plant–herbivore interaction.

Atmospheric chemists have discovered and characterized the vast emission of MeOH from plant canopies (Holzinger *et al.*, 2000; MacDonald and Fall, 1993), but the functional consequences of the release have not been thoroughly explored, largely because the analytical procedures chemical ecologists commonly use do not detect this abundant volatile. Real-time quantification of MeOH by PTR-MS revealed a pattern of release from *N. attenuata* that has been observed previously in *Quercus ilex* L. (Holzinger *et al.*, 2000), i.e. a diurnal increase, which decreases at night and is punctuated by a large increase at dawn (Figure 2 and Figure S1). This pattern correlates with the diurnal pattern of *NaPME* transcript accumulation, and suggests a release of MeOH through the stomata during the light phase. The large release at dawn, which is clearly associated with the initiation of the light phase (Figure S1), may result from the concentration of MeOH either in the dew on the leaf surface or in the apoplast. MeOH may concentrate in the apoplast when the stomata are closed during the dark phase and may be released when the stomata open at dawn.

Superimposed on this diurnal pattern is a substantial increase in MeOH emissions when plants are attacked by *M. sexta* larvae. The release is elicited by surprisingly small amounts of damage. Five neonate *M. sexta* larvae remove

less than 1 cm^2 of leaf area during the first 24 h of attack (Schmidt *et al.*, 2005), which is <1% of the total leaf area. This amount of damage was sufficient to increase the release of MeOH by 30%. This substantial whole-plant MeOH emission exceeded the release of *E*-2-hexenal. The increased rate of MeOH release in attacked plants is sustained throughout the night, albeit at a lower rate (Figure S1). Recently, *Euphydryas aurinia* larvae feeding on *Succisa pratensis* leaves were reported to elicit the release of large amounts of MeOH within 1 h of attack; this increase was sustained 24 h later, a result consistent with our findings (Peñuelas *et al.*, 2005). Although the mechanical damage inflicted by larval feeding increases the release rate, the increase is primarily elicited by herbivore attack. Such elicitation can be simulated by applying larval OS to mechanical wounds. Thus the induced increase of MeOH emissions can be regarded as an herbivore-specific quantitative signal.

Larval OS are known to contain potent elicitors (FACs) that reconfigure a plant's wound response and elicit a delayed systemic release of terpenoid VOCs that play important roles in defense signaling in *Zea mays*, *Phaseolus lunatus* and *N. attenuata* (Alborn *et al.*, 1997; Halitschke *et al.*, 2001; Koch *et al.*, 1999). Neither the two most abundant FACs, *N*-linolenoyl-L-Gln and *N*-linolenoyl-L-Glu, nor any of the other known herbivore-specific elicitors were able to induce MeOH emission at rates higher than those induced by wounding. Similarly, neither JA nor ethylene, both of which are known to be involved in stress-related volatile emission (Halitschke and Baldwin, 2003a; Schmelz *et al.*, 2003), increased the MeOH emissions of *N. attenuata* leaves (Table 1).

By manipulating *M. sexta* OS with buffers of different pH values, we demonstrate that pH elicits herbivore-induced MeOH emission (Figure 3). The pH of lepidopteran larvae midgut fluids is known to be extraordinarily high and to remain high despite continuous intake of leaf material with a substantially lower pH (Schultz and Lechowicz, 1986). Strong associations between the high-pH midgut fluids, which are energetically demanding to sustain (Karowe and Martin, 1993), and host plant use suggests that alkaline midguts are adaptive (Appel and Maines, 1995; Clark, 1999). The midgut fluids of insect species feeding on Solanaceous host plants or other taxa with high tannin contents are commonly strongly alkaline. A high midgut pH is thought to influence the toxic and anti-nutritive effects of plant defense chemicals by influencing the oxidative state of phenolics and other allelochemicals and reducing the formation of protein–tannin complexes (Barbehenn and Martin, 1998; Johnson and Felton, 1996). In addition, the quality and quantity of protein extracted from ingested leaves are thought to improve and increase under alkaline conditions (Felton and Duffey, 1991). The observation that a high pH elicits a release of MeOH that suppresses the host's defense responses in the *N. attenuata–M. sexta* interaction adds an additional function to the role of alkalinity in midgut fluids.

Applications of MeOH in quantities conservatively estimated to be released from attacked plants doubled the weight gain of *M. sexta* larvae feeding on these plants compared to that of those feeding on water-treated plants (Figure 5). The MeOH-elicited increase in larval performance correlated with a decrease of TPI activity in leaves (Figure 5, inset). This increase in larval performance was comparable to that observed in previous research with a genotype of *N. attenuata* whose TPI expression was impaired (Glawe *et al.*, 2003; Zavala *et al.*, 2004). How MeOH suppresses TPI activity remains unknown. TPI mRNA accumulates after methyl jasmonate treatment or *M. sexta* attack, leading to increased TPI activity and increased resistance to *M. sexta* (Zavala *et al.*, 2004). The TPI protein is post-translationally modified to its active form by two proteases, one of which is known to be regulated by JA signaling (Horn *et al.*, 2005). MeOH exposure may influence any of the transcriptional or post-translational steps involved in TPI elicitation.

Recently, a study on MeOH-responsive genes was conducted with *Arabidopsis thaliana* to obtain information about MeOH metabolism in plants; MeOH treatment was found to elicit large-scale responses in the transcriptome, some of which could be attributed to detoxification products of MeOH (Downie *et al.*, 2004). Elevated concentrations of CO_2, the last step of MeOH metabolism (Theodoridou *et al.*, 2002), increase the susceptibility of soybean to Japanese beetle attack, probably as a result of increases in leaf sugar contents (Hamilton *et al.*, 2005); this suggests that MeOH release may effect metabolic changes that influence the susceptibility of plants to herbivores. Whether the OS-elicited MeOH release functions as an indirect defense, as has been demonstrated for the herbivore-induced releases of GLVs and terpenoid VOCs (Kessler and Baldwin, 2001; Turlings *et al.*, 1990) and suggested by Peñuelas *et al.* (2005), will require additional study. As MeOH has a comparably long half-life in the atmosphere, differences in emission rates may be detected at substantial distances from an attacked plant. Clearly more information is needed about the responses elicited by the MeOH released from pectin demethylation before its role in plant–herbivore interactions can be fully appreciated.

The pH-dependent release of MeOH was not unexpected, as changes in cell-wall pH are known to change the activity of such wall proteins as expansins and PMEs, which in turn alter the rigidity of expanding cell walls (Bordenave and Goldberg, 1994; Cosgrove, 1997). Two lines of evidence suggest that induction of cell-wall-bound PMEs contributes significantly to the OS-elicited MeOH release: the demethylation of pectin polymers, as revealed by ^{1}H-NMR of pectin polymers before and after OS elicitation (Table 2 and Figure S3), and the OS-elicited increase in PME activity (Figure 4c) and mRNAs (Figure 4b). In mung bean, *Vigna*

radiata (L.), pH control of PME activity was found to be stronger in cell-wall-bound enzymes than in soluble enzymes (Bordenave and Goldberg, 1994), with the highest activity reported under slightly alkaline conditions. Because crude protein extracts were used to measure PME activity in this study, the intracellular origin of the enzymes remains unknown.

Silencing the expression of cell-wall-bound PMEs would allow the involvement of PMEs in the OS-elicited MeOH release to be tested directly. Analysis of the OS-elicited increase in *NaPME* mRNA (Figure 4c) revealed that the transcriptional and post-translational responses were too slow to account for either the OS-elicited increase in activity (Figure 4c) or the OS-elicited MeOH release (Figure 2 and Figure S1). In two studies, changes in the MeOH production were shown to be related to the genetic manipulation of PME expression (Frenkel *et al.*, 1998; Hasunuma *et al.*, 2003), supporting the association of PME with herbivore-induced MeOH emission.

The second well-described source of MeOH emission from plants is the activity of L-isoaspartyl methyltransferase, which is primarily localized in seeds. Certain plant species display activity in vegetative tissues (Thapar *et al.*, 2001). Treatment with ABA, as well as dehydration and salt stress, is known to increase L-isoaspartyl methyltransferase mRNA and activity, demonstrating the environmental responsiveness of PIMT (Mudgett and Clarke, 1994), but application of ABA to wounds of *N. attenuata* leaves did not increase MeOH emissions. To our knowledge, herbivore-induced regulation of PIMT has not been investigated so far. As the highest L-isoaspartyl methyltransferase activity reported from leaves is <50 pmol min^{-1} g^{-1} FM, which would be undetectable by our PTR-MS measures, we conclude that the L-isoaspartyl methyltransferases are unlikely to account for the significant difference in MeOH emission observed after herbivore attack in comparison with control plants.

In summary, we have much to learn about the role played by MeOH emission in plant–herbivore interactions. The release of MeOH appears to function as a quantitative signal, which indicates the importance of understanding the dynamics of MeOH metabolism in plants. More generally, this study highlights the value of studying plant traits in the context of the plant's ecological interactions, and cautions against use of the epithet 'waste product'. Plant traits can attain functional significance at many levels in the hierarchy of interactions in which all biological phenomena are embedded.

Experimental procedures

Plant growth

For whole-plant MeOH measurements, seeds of an inbred line of *Nicotiana attenuata* Torr. ex. Wats. (synonymous with *Nicotiana torreyana* Nelson and Macbr.) were germinated in smoke-treated soil. Seedlings in the four-leaf stage were transferred to communal boxes and later to 1 l individual hydroponic containers as previously described (Baldwin and Schmelz, 1994). All other experiments were performed with seedlings germinated on Petri plates as described by Krügel *et al.* (2002), transplanted after 10 days into potting soil, and grown for an additional 2–3 weeks. All plants were grown under high-pressure sodium lamps (800–1000 μmol m^{-2} sec^{-1} photosynthetically active radiation) with a 16 h/8 h light/dark cycle, at 28°C and 65% humidity during the light cycle. Experiments were conducted with late rosette-stage plants.

Methanol analysis

MeOH was measured in the headspace of either whole plants or single leaves. For whole-plant measurements, hydroponically grown plants were placed into 1 l desiccators 24 h before the experiments began. The hydroponic solution and the roots were separated from the shoot by plastic wafers, so that the remaining air-filled volume of the desiccator was about 780 ml. During the experiment, the individual desiccators, each containing either an untreated control plant or a plant attacked by five first-instar *M. sexta* larvae (three replicates each), were connected to the input valve of the proton-transfer-reaction mass spectrometer (PTR-MS; IONICON GmbH, Innsbruck, Austria) with a six-port sampling valve (Knauer, Berlin, Germany) and supplied with compressed air filtered through a zero-air generator (Whatman, Maidstone, UK) at a flow rate of 100 ml min^{-1} (Figure 1a). The desiccators were sampled sequentially for 10 min each at a rate of 15 ml min^{-1} for 40 h. The airflow through the other five chambers created positive pressure and prevented contamination of the chamber air by ambient air. During the measurements, the 16 h/8 h light/dark cycle was maintained by a single high-pressure sodium lamp installed above the desiccators (supplying 400–800 μmol m^{-2} sec^{-1} photosynthetically active radiation); the light regime during the second night of the second experiment was changed to continuous light to determine whether the MeOH emission that occurred at the onset of the light phase depended on light levels or was under circadian control (Figure S1). For single-leaf measurements, the leaves were placed into a 275 ml glass chamber, through which air was passed at a rate of 275 ml min^{-1}; a portion (15 ml min^{-1}) of the air was analyzed by PTR-MS (Figure 1b).

MeOH was measured online using a calibrated version of the PTR-MS equipped with a heated gas inlet capillary system and operated with a drift tube pressure of 2 mbar. The sample gas was continuously introduced into the chemical ionization cell, in which VOCs with proton affinities greater than water (proton affinity of H_2O: 166.5 kcal mol^{-1}) were ionized by proton transfer from H_3O^+ and subsequently analyzed in the quadrupole mass analyzer of the instrument. The intensities of selected mass ions (m/z) were monitored with dwell times of 200 msec for MeOH (m/z 33), 100 msec for m/z 37, and 1 sec for all other ion species (m/z 31, 57, 81, 83 and 137) to provide an overall time resolution of 10 data points per min when all masses of interest were recorded. As ionization by proton transfer is relatively soft, fragmentation of the target molecule rarely occurs. All (99%) ionized MeOH is recorded as the parent ion ($M + 1$, $m/z = 33$), obviating the concern that ionization-induced fragmentation confounds the association between the measured mass spectra and the actual headspace composition, as frequently occurs in conventional EI mass spectrometry in the analysis of light gases. As outlined by Lindinger and Hansel (1997) and Lindinger *et al.* (1998), a simple relationship exists between the experimentally measured PTR-MS mass spectral intensities (in counts per second) and the absolute concentration in the gas stream. The temperature in the inlet system and the

drift tube was 80°C. We assumed a rate constant for the proton transfer reaction of 2×10^{-9} cm^3 sec^{-1} and a residence time of the reactants in the drift tube of 105 μsec based on the measured vacuum and the manufacturer's specifications. While the source delivers a pure ion stream of H_3O^+ ions, we monitored the formation of $(H_2O{\cdot}H_3O)^+$ clusters ($m/z = 37$), which may become important at high levels of humidity in the inlet gas stream when leaves have been wounded. These ion clusters could change the reaction conditions in the drift tube and confound the quantification of MeOH. However, we found that the proportion of these clusters remained below 5% of the H_3O^+ ions under all conditions. A standard curve for MeOH was obtained by repeated injections of 0.25, 0.35, 0.5 and 1 μl MeOH in a 1 ml volume with a gas-tight syringe into the flow chamber. The calibration curve was based on integration of the total MeOH peaks.

Methanol elicitation

For single-leaf measurements, the petiole of the youngest, fully expanded, rosette leaf was detached from the plant with a razor and immediately transferred into a 2 ml Eppendorf tube filled with distilled water. The leaf was inserted into the flow chamber and the MeOH emission was measured for 5 min as control emission. The leaf was then wounded with a fabric pattern wheel that created three rows of punctures on each side of the mid-rib; MeOH emission was measured for an additional 5–10 min. The test solutions were subsequently applied to the puncture wounds, and MeOH release was again measured for an additional 5–10 min. The integrated areas of the two measurement periods after wounding were converted into μmol MeOH released and expressed as rates of wound and total MeOH emission. To calculate the amount of induced MeOH, the wound-elicited emission was subtracted from the total emission (Figure 3, inset).

Simulation of herbivore attack by mechanical damage and application of *M. sexta* larvae OS is precise and allows the effects of wounding to be differentiated from those that are herbivore-specific. To identify the signals in larval OS that were responsible for the MeOH release, 20 μl of the following solutions were applied to the puncture wounds: water; *M. sexta* OS collected from larvae reared on *N. attenuata* leaf diet, which was freeze-dried to remove internal MeOH and diluted 1:1 v/v with water (OS); 0.06 mM *N*-linolenoyl-L-Gln and 0.17 mM *N*-linolenoyl-L-Glu in 0.05% Triton X100 (FACs); ion-exchanged OS eluted four times through ion-exchange columns containing 400 mg of the basic ion-exchange resin Amberlite IRA-400; 0.04 mM jasmonic acid (Cayman, Ann Arbor, MI, USA) in water; 0.05 mM salicylic acid in 0.1% Triton X100; 0.5% Triton X100; 0.69 mM 2-chloroethylphosphonic acid in 1 N H_3PO_4; 40 mM H_2O_2; 5 μg μl^{-1} chitosan in acidic water; 5 μg μl^{-1} polygalacturonic acid (PGA) in water; 50 U ml^{-1} glucose oxidase in 20 mM sodium phosphate buffer, pH 6.5; OS boiled for 20 min prior to application; OS treated with proteinase K (USB, Amersham Bioscience, Freiburg, Germany) with a final concentration of 100 μg ml^{-1} in the reaction mixture; 0.1 M sodium phosphate buffer (pH 6–9); 0.2 M sodium phosphate buffer/0.1 M citric acid (pH 5); 0.1 M MES (pH 5.5 and pH 6.5); 0.1 M MES/Tris mixtures (pH 6.5 and pH 7.5); 0.1 M Tris (pH 7.5 and pH 9); 50 mM and 250 mM ABA. All chemicals used were obtained from Sigma (Taufkirchen, Germany) unless otherwise stated.

Pectin methylesterase activity assay

PME activity was measured in protein extracts from the leaf lamina of *N. attenuata* control plants and after wounding and application of water or application of *M. sexta* OS as described above. For the quantification of PME activity, a gel diffusion assay described by Downie *et al.* (1998) and modified by Bourgault and Bewley (2002) was used. Briefly, leaf samples were frozen in liquid nitrogen and homogenized in 1 ml extraction buffer (1 M NaCl, 0.1 M citrate/0.2 M sodium phosphate, dibasic, 2.5 M PMSF, 10 μM leupeptin, pH 7). After centrifugation (16 000 *g*, 4°C), 10 μl aliquots of supernatant were loaded into 2 mm wells in a 2% w/v agarose gel containing 0.1% pectin (Sigma) and 0.5 mg ml^{-1} gelatin (Merck, Darmstadt, Germany). The gels were incubated for 16 h at 26°C, rinsed with water, and stained with 0.01% ruthenium red (Sigma) for 45 min. The diameter of each stained zone (representing demethylated pectin) was measured to the nearest 0.1 mm. Units of PME activity were calibrated with orange peel PME (Sigma). One unit releases one microequivalent of acid from pectin per min at pH 7.5 and 30°C (16.66 nkatal). PME activities are expressed as units per mg protein as determined by the Bradford (1976) method . Adding MeOH to the protein extracts to produce a final concentration of up to 2.5% MeOH in the extract did not change measures of protein concentration or PME activity (data not shown).

^{1}H-NMR measurements of pectin demethylation

Leaves were elicited, and 30 min later, leaf samples were weighed, frozen in liquid nitrogen, and stored at −80°C. Pectin was isolated using the method of Bédouet *et al.* (2003). Briefly, the samples were homogenized at 4°C with a commercial homogenizer (Ultra-Turrax T25; IKA Labortechnik, Staufen, Germany) in 50 mM AcONa buffer and filtered through Whatmann number 3 paper. The insoluble fraction was washed twice with $CHCl_3$:MeOH 1:1 v/v. The fraction was extracted with 50 mM HCl at 70°C. The extraction was stopped by bringing the solution to pH 5 with 1 M NH_4OH. After centrifugation (16000 ***g***, 20°C), the supernatant was extracted with 3 volumes of EtOH at 4°C overnight. Pectin was collected by centrifugation (16000 ***g***, 4°C) and dehydrated before dissolving in D_4-MeOH. NMR measurement was performed on a Bruker DRX 400 NMR spectrometer, operating at 500, 13 MHz for ^{1}H. The sample was measured in D_4-MeOH. Chemical shifts are given in δ values with reference to tetramethylsilane (TMS) as an internal standard, with coupling constants in Hz. After the first measurement at 80°C, deuterated sodium hydroxide (NaOD) was added to the NMR tube and additional scans were recorded to measure the loss of methylesters upon saponification (Figure S3).

Analysis of NaPME *mRNA accumulation*

Quantitative real-time PCR assays were used to quantify transcript accumulation of *NaPME*. The sequence of the putative *N. attenuata PME* (*NaPME*; Figure S6; accession number DQ115979) was obtained by PCR using primers designed from the nucleotide sequence alignment of several PME from Solanaceaous plants including the PME of *Solanum tuberosum* (BQ505674-1), which showed the largest up-regulation in a replicated microarray analysis comparing *M. sexta*-attacked and untreated control plants (Schmidt *et al.*, 2005). Total RNA was extracted from treated leaves of *N. attenuata* plants 45, 90 and 135 min, and 4, 8, 12 and 24 h after elicitation. The youngest fully expanded leaf was wounded with a pattern wheel and 20 μl of water or *M. sexta* OS diluted 1:1 v/v with deionized water were applied to the fresh wounds. cDNA was synthesized from 20 ng of total RNA as described by Schmidt *et al.* (2005).The following primer and fluorescence dye-labeled probe combination was used: PME_F1 (GGAGTGGTTGTCCGCTGG),

PME_R1 (CCTCCGACACCGTCCTGA) and PME_P1 (FAM-CTGCAGTCTTCGACGGTGAGGCC-TAMRA; Figure S6). Real-time PCR was performed on an SDS7700 (Applied Biosystems, Darmstadt, Germany) using the qPCR™ reagent kit (Eurogentec, Seraing, Belgium); for a detailed description see Halitschke and Baldwin (2003a) and Schmidt *et al.* (2005).

Herbivore performance

The average induced MeOH emissions of an attacked plant were 0.063 μl min^{-1} g^{-1} FM from hydroponically grown plants with an average shoot mass of 4.5 g. Hence, herbivore attack typically elicits a release of 103 μl MeOH over 6 h. Despite the long half-life of MeOH in the atmosphere, convection prevents this quantity of MeOH from being taken up completely by an attacked plant or an adjacently growing plant. To mimic realistic exposure to MeOH, we sprayed soil-grown plants that had an approximate shoot mass of 7 g three times during the light phase with 2 ml of a 5% aqueous MeOH solution that delivered a total of 100 μl MeOH to the plant surface, less than the calculated exposure during herbivore attack. Control plants were sprayed with 100% water. At the end of the second day, when the foliage was completely dry, freshly eclosed *M. sexta* larvae were placed on the youngest fully expanded leaf and their masses determined on days 2, 4, 6, 9 and 11. At the onset of feeding, leaf samples of the youngest fully expanded leaf and its orthostichous leaf from an additional set of identically treated plants were harvested, placed in liquid nitrogen, and analyzed for trypsin protease inhibitor activity as described by van Dam *et al.* (2001). To examine the impact of exogenously applied MeOH on the activity of the protein extracts, MeOH was applied to 200 μl protein extract, equivalent to 100 mg leaf material, resulting in final concentrations of 0.025‰, 0.25‰, 0.25% and 2.5% MeOH in the protein extract. The highest concentration of MeOH in the protein extract (2.5%) decreased TPI activity significantly compared to the water-added control (*t*-test; $P < 0.05$). This concentration is equivalent to an MeOH addition to the protein extract that is three times that which was added to the corresponding leaf material in the experiment.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Pattern of MeOH emission from attacked and control *N. attenuata* plants over 40 h of continuous measurements.

Mean μmol MeOH min^{-1} g^{-1} FM ± SE released by control plants (open symbol) and plants that were under attack by five neonate *M. sexta* larvae (filled symbol). MeOH emission was analyzed by proton-transfer-reaction mass spectrometry (PTR-MS) configured with a six-port valve (Figure 1a), which sampled the headspace for 10 min of each of six individual plant chambers for 40 h. Open and dark bars designate day and night periods, respectively. The two night cycles differed in their light regime indicated by the black (lights off) and gray (lights on) bar. Note the high rates of MeOH emission from both attacked and control plants at the beginning of the light phase.

Figure S2. Induced MeOH emissions increase with increasing pH.

Mean μmol MeOH min^{-1} g^{-1} FM ± SE released from single *N. attenuata* leaves after standardized puncture wounding and application to the wounds of 20 μl of A. 0.1 M phosphate buffers with the indicated pH values and B. 0.1 M MES, 0.1 M MES/TRIS, and 0.1 M TRIS with the indicated pH values. Wound-induced MeOH emission (white bars) and MeOH emissions elicited after the test solutions were applied to the puncture wounds (black bars) are presented.

Figure S3. ^{1}H NMR spectra of pectin extracts before and after saponification.

NMR measurement was performed on a Bruker DRX 400 NMR spectrometer, operating at 500, 13 MHz for ^{1}H. The samples were measured in D_4-MeOH. Peaks are labeled according to their representative chemical shifts given in δ values in reference to a TMS as internal standard with coupling constants in Hz. An example of a wounded leaf is presented. After the first measurement at 80°C, NaOD was added to the NMR tube and further scans were recorded to measure the loss of methylesters upon saponification. Note that the signals of esterified MeOH and AcOH disappear after saponification and that the free MeOH increases.

Figure S4. A representative PTR-MS scan of MeOH after wounding and OS application.

Relative MeOH concentration (counts sec^{-1} of m/z 33) in the emission of a single leaf. The emission of an untreated control leaf is shown before time = 0 min, the time of treatment. The induced MeOH emission, by wounding, and application of 20 μl of OS of *Manduca sexta* (1:1 diluted with water) reached a maximum about 15 min after the treatment and returned to control levels at about 120 min.

Figure S5. Diurnal expression of *NaPME* in *N. attenuata*.

Relative expression ratio ±SE in arbitrary units of *NaPME* transcripts analyzed by RT-PCR. cDNA was transcribed from 5 replicated untreated *N. attenuata* plants (open triangles) for each time point. Samples were harvested over a 24-h period, with the samples at 9:00 am harvested on the second day. The light/dark regime was set to 16 h:8 h starting at 6:00 a.m.

Figure S6. *Nicotiana attenuata* partial mRNA for pectin methylesterase

Sequence information on *NaPME* (Accession No. DQ115979). Forward and reverse primers (PME_F1 and PME_R1) and probe (PME_P1) used for RT-PCR assays are underlined.

This material is available as part of the online article from http://www.blackwell-synergy.com

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Manuscript II

Deciphering the role of ethylene in plant-herbivore interactions

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This manuscript reviews the available literature on herbivore-induced ethylene emissions and the insect-derived elicitors that trigger a plant to release ethylene. Furthermore, it provides a thorough overview of the role of ethylene regulating direct and indirect defense responses of plants and summarizes the impact of ethylene-dependent defense responses on herbivore and plant performance. Finally, the manuscript emphasizes the usefulness of mutant plants for investigating ethylene's function in herbivore-plant interactions. I selected the cited literature for this article; Ian T. Baldwin and I outlined and wrote this article together.

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Deciphering the Role of Ethylene in Plant–Herbivore Interactions

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Abstract

Most plants emit ethylene in response to herbivory by insects from many different feeding guilds. The elicitors of these ethylene emissions are thought to be microorganisms or oral secretion-specific compounds that are transferred when the attacking insect feeds. To find the receptors for these elicitors and describe the signaling cascades that are subsequently activated will be the challenge of future research. Past experiments on the function of herbivore-induced ethylene, which were biased toward the use of chemical treatments to manipulate ethylene, identified seven ethylene-dependent defense responses. In contrast, a genetic toolbox that consists of several mutants has rarely been used and to date, mutants have helped to identify only one additional ethylene-dependent defense response. Ethylene-dependent responses include the emission of specific volatile organic compounds as indirect defense, the accumulation of phenolic compounds, and proteinase inhibitor activity. Besides being ethylene regulated, these defenses depend strongly on the wound-hormone jasmonic acid (JA). That ethylene requires the concomitant induction of JA, or other signals, appears to be decisive. Rather than being the principal elicitor of defense responses, ethylene modulates the sensitivity to a second signal and its downstream responses. Given this modulator role, and the artifacts associated with the use of chemical treatments to manipulate ethylene production and perception, future advances in the study of ethylene's function in plant–herbivore interactions will likely come from the use of signaling mutants or transgenic plants. It will be exciting to see if adaptive phenotypic plasticity is largely an ethylene-mediated response.

Key words: Defense response; Ethylene emission; Herbivory; Ethephon; 1-MCP; Genetic manipulation; Mutants; VOCs

Phytohormones regulate almost all developmental processes in plants, from germination, to growth and differentiation, to their carefully timed senescence. Hormones transform external stresses into internal responses and allow a plant to adjust its phenotype to prevailing environmental conditions.

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Although ethylene is known to be involved in most developmental processes and has been shown to be the signal mediating the response to several external stimuli, for example, pathogens and drought, it is another phytohormone, jasmonic acid (JA), that is thought to be the main mediator of responses to herbivore attack (Halitschke and Baldwin 2005).

The observation that herbivore attack elicits an ethylene burst was made as early as 1950, when an elegant experimental procedure, the triple response

of etiolated pea seedlings, was used to show that infested rose leaves produced a physiologically active ethylene release (Williamson 1950). In five subsequent decades of research, the list of plant taxa shown to emit ethylene in response to herbivore attack has grown long, but our understanding of the hormone's function in the interaction has grown only incrementally. Plants, as sessile organisms, can use ethylene as a volatile hormone to interact either with distant plant parts or with other plants in their proximity. Thus, it is surprising that research on how ethylene regulates a defense response in plant–herbivore interactions is still in its infancy.

One explanation may be the technical challenges of manipulating ethylene production, accumulation, and perception with chemical treatments. In addition to the toxic byproducts from ethephon and silverthiosulfate applications, phosphoric acid and silver, determining the right timing and concentration of these and other chemicals to manipulate ethylene signaling during complicated processes such as the wound response is clearly difficult. Ethylene and 1-methylcyclopentene (1-MCP) are gases readily diffusible through plant tissue, but their ability to provide specific control of ethylene-dependent defense responses is limited. 1-MCP has been successfully used to regulate ethylene-dependent postharvest processes of fruits and vegetables, and the physiological and biochemical responses are well known (Watkins 2006). Given that the maintenance of product quality, which involves ripening, softening, and senescence, is in some cases achieved by sequential application of 1-MCP and ethylene and depends strongly on timing (Watkins 2006), it is not surprising that the use of 1-MCP is limited when complex and faster responses during herbivory need to be simulated. Wounding of 1-MCP–treated detached fruits is known to cause unexpected responses due to feedback regulation of ethylene biosynthesis. During the wound-induced ethylene biosynthesis in *Prunus persica*, 1-MCP had no effect on 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase activity but inhibited the accumulation of its transcripts while increasing the transcript accumulation of ACC synthase (Mathooko and others 2001). Ethylene regulation during herbivory on a whole-plant level is likely to be multifaceted and thus different from the ethylene biosynthesis that occurs during developmental processes.

One example of ethylene's role during a developmental program, which implies ecological interactions, is the plant–insect mutualism pollination. Initiation of post-pollination processes, such as the regulation of floral scent and flower abscission, can be ascribed to the ethylene burst that occurs after pollination and has been successfully mimicked (Llops-Tous and others 2000; Negre and others 2003; Patterson and Bleecker 2004). Research advances have been driven by a clear definition of the traits to be observed. Such clarity with regard to response variables is lacking in studies that investigate the function of ethylene in plant–herbivore interactions. An interdisciplinary approach combining molecular, biochemical, and ecological levels of analysis, similar to that used to understand the role that volatile organic compounds (VOCs) play in plant–herbivore and tritrophic interactions (Turlings and Ton 2006), will be required to fully understand the function of ethylene in plant–herbivore interactions.

Research into ethylene's biosynthetic pathway, and the signal transduction cascades that both elicit it and are elicited by its perception, has produced some stunning results (Klee 2004; Chae and Kieber 2005), and genetically transformed ethylene-insensitive plants have recently been used to further our understanding of competitive interactions among plants (Pierik and others 2004) and of the priming that occurs in plant–pathogen interactions (De Vos and others 2006). However, these molecular advances have yet to be fully incorporated into the study of the plant defense responses elicited by herbivore attack; most research still relies heavily on the use of exogenous chemical treatments to manipulate ethylene responses. Here we review the literature that illuminates the function of ethylene in plant–herbivore interactions and predict advances that will likely result from the use of ethylene perception and biosynthesis mutants in species whose plant–herbivore interactions have been particularly well studied.

Ethylene Emission and Herbivory

Elicitation of the Herbivore-induced Ethylene Emission

The fact that herbivory elicits an ethylene release has been recognized since the use of the triple response of etiolated pea seedlings to examine ethylene emanation of rose leaves that had been infected by several pathogens and the red spider mite *Tetranychus telarius* (Williamson 1950). Mite-infested tissue produced more ethylene than did healthy leaves, but the strongest emanation was caused by pathogen attack. In subsequent decades, numerous methods have been used to detect ethylene, but only two are still commonly used: either ethylene is concentrated in the headspace of infested tissues and subsequently measured by a gas

chromatograph equipped with a flame-ionization detector (GC-FID), or on-line measurements of attacked plants are conducted by photo-acoustic spectroscopy (PAS), with a laser light source adjusted to the critical wavelength to excite ethylene molecules (Table 1). These techniques enable researchers to demonstrate that almost all plant taxa emit ethylene when attacked by arthropod herbivores, that the ethylene release is not due solely to the damage caused by the attacking herbivore, and that attack from herbivores of many different feeding guilds elicits a response (Table 1).

Although mechanical wounding can result in increased basal ethylene release, research using pins to simulate the feeding damage inflicted by cotton fleahoppers and basswood thrips clearly demonstrated that the insect is required for a pronounced ethylene burst (Duffey and Powell 1979; Rieske and Raffa 1995). The wound-hormone JA, which is responsible for integrating several direct and indirect plant defense responses following herbivore attack (Halitschke and Baldwin 2005), and systemin, a peptide signal that functions upstream of the oxylipin pathway leading to JA (Schilmiller and Howe 2005), have been shown to increase ethylene emissions from tomato plants above levels observed in control plants. The induced quantities were proportional to the wound-induced amounts (O'Donnell and others 1996). Hence, the herbivore-induced ethylene burst is qualitatively different from wound-induced ethylene releases; only systemin, and not JA, has been shown to elicit the release of ethylene.

The fact that ethylene is released after attack by many different feeding guilds informed early research on the mechanisms of elicitation. Early studies with piercing-sucking insects regarded the influence of ethylene to be secondary and the insect to be the vector of a microbial elicitor. R. D. Powell and co-workers, working on the microbial colonization of cotton fleahoppers, were able to separate the wound response from the fleahopper attack and to correlate the abundance of microorganisms in the herbivore with the amount of ethylene induced (Duffey and Powell 1979; Grisham and others 1987). The origin of the ethylene-induced abscission of cotton flower buds caused by insect attack was ascribed to the wounds inflicted and salivary enzymes injected, and to the microorganisms that invaded the wounds (Martin and others 1988). Studies of the ethylene emissions of onion plants, *Allium cepa*, in response to *Thrips tabaci* demonstrated that thrip extracts applied to mechanical wounds in onion plants mimicked the ethylene emission of thrip-attacked plants (Kendall and Bjostad 1990). Although whole-insect extracts were found to contain the elicitors in thrips, only the oral secretions (OS) of the beetle *Leptinotarsa decemlineata* were required to elicit the ethylene burst in attacked *Solanum tuberosum* and *Phaseolus vulgaris* plants (Kruzmane and others 2002; Steinite and others 2004). The compounds in OS, which themselves elicit the ethylene response in interactions with thrips and with beetles, are unknown.

In two well-studied plant–herbivore systems, *Manduca sexta–Nicotiana attenuata* and *Spodoptera exigua–Zea mays*, herbivore-specific defense responses that are regulated by JA, such as the emission of volatile organic compounds (VOCs) and trypsin proteinase inhibitor (TPI) activity, are elicited by fatty acid–amino acid conjugates (FACs) present in OS (Alborn and others 1997; Halitschke and others 2001). The *M. sexta*–induced ethylene emissions from *N. attenuata* plants are elicited by FACs, the same OS-derived elicitor that increases JA accumulation (C. C. von Dahl, R.A. Winz, R. Halitschke, F. Kühnemann, K. Gase and I.T. Baldwin, unpublished). However, although JA and ethylene are both OS dependent, they are regulated independently of one another. *N. attenuata* as-*lox3* plants are retarded in their JA accumulation following herbivory due to the anti-sense expression of the specific lipoxygenase (NaLOX3) that supplies fatty acid hydroperoxides to the oxylipin cascade (Halitschke and Baldwin 2003). Oral secretion–induced ethylene emissions of these as-*lox3* plants are not different from those of wild-type (WT) plants, and inverted repeat *ACO* construct (ir-*aco*) plants, which do not release ethylene after herbivore attack due to the silencing of Na*ACO* by RNAi, show an OS-induced JA burst similar to that of WT plants (C.C. von Dahl and I. T. Baldwin unpublished). This suggests that an upstream signal, which is activated by the perception of FACs, modulates the biosynthesis of these hormones. Possible candidates are mitogen-activated protein kinases (MAPKs), which regulate ethylene biosynthesis during the wound-response by altering the stability of ACC synthase (ACS) (Chae and Kieber 2005).

In contrast, the OS-specific responses of *Vigna unguiculata* and *P. vulgaris*, which include JA accumulation and the release of ethylene, were elicited by applying specific fragments of the plants' ATP synthase (called inceptins) (Schmelz and others 2006). Inceptins elicit responses in bean and cowpea but not in maize and tobacco, which suggests that the elicitors of ethylene release vary structurally from peptide fragments of plant proteins digested by

Table 1. Ethylene Emission during Herbivore Attack

Plant species	Insect species	Ethylene	Source	Emission	Detection	Reference
Gossypium hirsutum	*Pseudatomoscelis seriatus* (Het)	1.46 nmol g^{-1} FM h^{-1}	Cut bud	Accumulation	GC-FID	Duffey and Powell 1979
Vigna unguiculata	*Megalurothrips sjostedti* (Thys)	26.67 nmol g^{-1} FM h^{-1}	Cut peduncle	24 h accumulation	GC-FID	Wien and Roesing 1980
Malus domestica	*Phyllonorycter blancardella* (Lep)	0.19 nmol g^{-1} FM h^{-1}	Cut leaves	4 h accumulation	GC-FID	Kappel and others 1987
Allium cepa	*Thrips tabaci* (Thys)	0.001 nmol g^{-1} FM h^{-1}	Intact plant	24 h accumulation	GC-FID	Kendall and Bjostad 1990
Medicago sativa	*Therioaphis maculata* (Hom)	0.53 nmol g^{-1} FM h^{-1}	Cut trifoliolate	4 h accumulation	GC-FID	Dillwith and others 1991
Triticum aestivum	*Schizaphis graminum* (Hom)	1.6 nmol g^{-1} FM h^{-1}	Cut shoot	18 h accumulation	GC-FID	Anderson and Peters 1994
Hordeum vulgare (Morex)	*Diuraphis noxia* (Hom)	0.006 nmol $plant^{-1}$ h^{-1}	Intact plant	4 h accumulation	GC-FID	Miller and others 1994
	Schizaphis graminum (Hom)	0.012 nmol $plant^{-1}$ h^{-1}				
Tilia americana	*Thrips calcaratus* (Thys)	0.008 nmol g^{-1} DM h^{-1}	Intact seedling	8 h accumulation (presumed)	GC-FID	Rieske and Raffa 1995
		0.009 nmol g^{-1} DM h^{-1}	Cut seedlings			
Nicotiana attenuata	*Manduca sexta* (Lep)	0.29 nmol $plant^{-1}$ h^{-1}	Intact plant	Online	PAS	Kahl and others 2000
Hordeum vulgare	*Schizaphis graminum* (Hom)	0.28 nmol g^{-1} FM h^{-1}	Intact seedling	Accumulation	GC-FID	Argandoña and others 2001
	Rhopalosiphum padi (Hom)	0.21 nmol g^{-1} FM h^{-1}				
Phaseolus lunatus	*Tetranychus urticae* (Acar)	0.062 nmol $plant^{-1}$ h^{-1}	Intact plant	Online	PAS	Arimura and others 2002
Zea mays	*Spodoptera exigua* (Lep)	0.25 nmol g^{-1} FM h^{-1}	Intact plant	1 h accumulation	GC-FID	Schmelz and others 2003
Arabidopsis thaliana	*Pieris rapea* (Lep)	0.057 nmol g^{-1} FM h^{-1}	Cut shoot	48 h accumulation	GC-FID	De Vos and others 2005
	Myzus persicae (Hom)	0.044 nmol g^{-1} FM h^{-1}				

Herbivore-induced ethylene emissions have been measured for more than 50 years, either by allowing ethylene to accumulate in the headspace of an attacked tissue and subsequently quantifying it using gas chromatography flame-ionization detection (GC-FID) or real-time photo acoustic spectroscopy (PAS) analysis with a laser source tuned so as to excite ethylene molecules. Almost all plant taxa emit ethylene after attack by a variety of herbivorous insect species. The ethylene quantities given are estimates of the published maximum values after conversion into nmol g^{-1} FM h^{-1}or nmol $plant^{-1}$ h^{-1}, to facilitate comparisons.
Abbreviations: Lepidoptera (Lep), Thysanoptera (Thys), Heteroptera (Het), Homoptera (Hom), Acarina (Acar).

the attacking herbivores to FACs produced in the alimentary canals of herbivores. This specificity in the elicitors suggests strong selection for the ability to respond to specific attackers.

The Consequences of the Ethylene Burst for Herbivore and Plant Performance

Early research on ethylene emission during herbivory was motivated by the secondary effects such emission had on developmental processes, for example, flower bud abscission and inhibited internode elongation, which were initiated when ethylene was released after herbivore attack (Duffey and Powell 1979; Wien and Roesing 1980; Miller and others 1994). Inhibited internode and leaf blade elongation and increased flower bud abscission following herbivory were associated with the ethylene burst, which is known to mediate these phenomena independently of herbivore attack. It is, however, abundantly clear that herbivore attack results in dramatic changes in resource allocation within a plant, and the phenomena described above might well be caused by rerouting resources to the roots and thereby skirting other carbon sinks. Recently, FACs in *M. sexta* OS were found to downregulate an SNF1 kinase in *N. attenuata* via a JA-independent pathway. The suppression of the SNF1 kinase alters allocation, sending recently fixed carbon to the roots, where it is bunkered for later use during regrowth after the herbivore has left the plant (Schwachtje and others 2006). Additionally, JA-induced defense compounds are known to be costly in terms of growth and seed production, especially in herbivore-free environments, as has been demonstrated for *N. attenuata* in its natural habitat (Baldwin 1998). These herbivore-induced changes in resource allocation influence plant growth and profoundly alter herbivore performance, not only by changing the suite of secondary metabolites that function as defenses (see below), but also by changing the primary metabolites that determine the nutritional value of a plant for an herbivore.

Herbivore bioassays provide the most direct means of determining whether ethylene-mediated responses alter herbivore performance on plants. In a study that examined the performance of *Myzus persicae* on *Solanum lycopersicum* exposed to a variety of different elicitors, only MeJA and BTH treatments slowed aphid population growth, whereas aphid counts were highest on ethephon-treated tomato plants (Boughton and others 2006). Unfortunately, this study lacks an analysis of the aphid population growth in response to different ethephon concentrations. Plant growth and flower abscission showed a dose-dependent response, indicating that ethylene activity depended on concentration. This relationship is also likely to affect both the plant's response and the aphids' subsequent performance. Contrasting results were obtained for *Arabidopsis thaliana* with regard to the population growth of two phloem-feeding aphids, *Myzus persicae* (a generalist) and *Brevicoryne brassicae* (a specialist); the population of both species was the same on ethylene-insensitive *etr1* and WT plants (Mewis and others 2005). Comparing the performance of the generalist *Spodoptera littoralis* with that of the specialist *Plutella xylostella* on mutant *Arabidopsis thaliana* plants affected in ethylene signaling, *hookless 1* (*hls1-1*) and *ein2-1*, revealed that the mutants were resistant only to the generalist herbivore (Stotz and others 2000).

The treatment of an insect-resistant *Zea mays* variety with $CoCl_2$, aminoethoxyvinylglycine (AVG), or 1-MCP increased both the feeding damage inflicted by the generalist *Spodoptera frugiperda* and the herbivore's relative growth rate in comparison to untreated control plants (Harfouche and others 2006). This susceptibility was attributed to an ethylene-responsive cysteine proteinase, Mir1-CP, which was only found in the resistant variety. The insect-susceptible *Z. mays* variety was not able to accumulate the proteinase; nor did the inhibition of ethylene-signaling change herbivore resistance (Harfouche and others 2006). Thus, ethylene signaling is involved in corn's activation of resistance to *S. frugiperda*. This is in contrast to the results obtained with *Arabidopsis*: there the growth rate of the generalist *S. exigua* decreased on *etr1* plants (Mewis and others 2005; Harfouche and others 2006). A detailed analysis of the effects of ethylene on the traits that are responsible for herbivore resistance is required to understand the reasons behind these ethylene-mediated changes in herbivore performance.

Herbivore-induced Ethylene and the Defense Response of Plants

Defenses can be constitutive or inducibly expressed, and indirect or direct in their mode of action, and they are further differentiated according to their mechanism, which may be mechanical, chemical, or proteinaceous. They often target features that are common among different insect attackers, for example, the digestive or nervous system, or the mobility of the insect. Indirect defenses— for example, emitted VOCs or extrafloral nectar—

function not by targeting the attacker but by attracting parasitoids or predators of insect herbivores, and these finally kill the herbivore.

Ethylene has been shown to elicit a battery of different defensive proteins. Polyphenoloxidase (PPO) and peroxidase (POD) are enzymes that are thought to form quinines, which subsequently react with nucleophilic side chains of amino acids leading to protein cross linkage. Quinones are thought to inhibit digestion in the insect gut. Aphid infestation of barley plants rapidly elicited hydrogen peroxide followed by soluble PODs that reached maximum levels after 25 min. Additionally, aphid infestation increased ethylene emission significantly after 1 h. When plants were exposed to 20 ppm ethylene, simulating the release that occurs in aphid-infested plants, hydrogen peroxide levels increased; but after 25 min, PODs had increased to levels only half of those induced by aphids (Argandoña and others 2001). Blocking ethylene biosynthesis and perception, using amino-oxyacetic acid and 1-MCP, respectively, as well as inhibiting protein synthesis, demonstrated that increasing the POD and PPO activity of *P. vulgaris* leaves in response to wounding and to the application of OS from *L. decemlineata* depended on ethylene production and was not caused by insect-derived enzyme activity (Steinite and others 2004). In tomato plants, PPO activity showed a threefold increase after MeJA application; yet no changes were observed after ethephon treatment. Ethephon, however, increased POD activity in a dose-dependent manner (Boughton and others 2006). When ethylene was first mentioned as a possible signal mediating the wound response of tomato plants, *Proteinase inhibitor 2* (*PIN2*) transcript accumulation was shown to depend on ethylene and JA synergistically (O'Donnell and others 1996). Proteinase inhibitors are another class of enzymes interacting with the herbivore's digestive system. When a resistant variety of *Z. mays* Mp708 was analyzed using several ethylene biosynthesis blockers and 1-MCP, resistance was shown to be ethylene dependent. Ethylene production and perception had no influence on herbivore performance in the susceptible variety Tx601. The protein activity and transcript accumulation of Mir1-CP, a cysteine proteinase, were also altered by ethylene signaling. This suggests ethylene is a component of the signal transduction pathway leading to resistance in maize and is mediated by cysteine proteinases (Harfouche and others 2006).

Although many of these defensive proteins are found in all plant taxa, small-molecular-weight secondary metabolites that function as defenses tend to be highly species or family specific. For example, glucosinolates (GS) are characteristic of Brassicaceae. Several mutants of *A. thaliana* were investigated with regard to their ability to elicit GS after attack by two aphid species and one lepidopteran herbivore. The GS response to all three insects required a functional NPR1 and ETR1. Although the *etr1* mutant did not significantly influence total GS, ethylene was required for the insect-induced accumulation of aliphatic GS. As caterpillar performance was retarded on *etr1* mutants compared to wild-type Col-1 plants, ethylene may have influenced more than just the regulation of GS (Mewis and others 2005). Nicotine accumulation (characteristic for *Nicotiana spp.*) depends on the activity of putrescine *N*-methyltransferase (PMT), the first committed and regulatory step in nicotine biosynthesis. Two studies investigated the interaction of JA and ethylene in PMT transcript accumulation and subsequent nicotine accumulation in *N. sylvestris* and *N. attenuata*. The application of ethylene and the use of 1-MCP and $AgNO_3$ revealed that the MeJA-induced accumulation of PMT genes and the production of nicotine are attenuated by ethylene (Shoji and others 2000; Winz and Baldwin 2001). This explains why the increased JA accumulation in response to OS compared to wounding did not increase nicotine in OS-treated leaves. Nicotine accumulation in response to OS was higher than wound-induced amounts when the plants had been pretreated with 1-MCP (Kahl and others 2000). Furthermore, synergistic effects between JA and ethylene have been found in the phenolic and terpenoid resin formation of Douglas fir, where the MeJA-induced defense responses are amplified by ethylene (Hudgins and Franceschi 2004).

A similar synergism between JA and ethylene was observed in the elicitation of terpenoid emission in *Z. mays*. Although pretreating plants with 1-MCP did not change the level of caterpillar-induced JA accumulation, it reduced the emission of sesquiterpenes and indole, both of which are thought to function as indirect defenses. Interestingly the emission of ethylene in response to feeding *S. exigua* was also retarded. The authors argue that reduced VOC production was due to blocked perception rather than changed ethylene emission (Schmelz and others 2003). Whereas exogenous ACC also enhances the production of JA-induced (*E*)- and (*Z*)-β-ocimene and (*Z*)-3-hexenyl acetate in lima bean plants, the application of ethylene, in addition to MeJA, to *N. attenuata* had no synergistic effect on *cis*-α-bergamotene emission (Kahl and others 2000; Horiuchi and others 2001). The activity of another oxylipin-derived signal is also synergized by ethylene. (*Z*)-3-hexenol induces the emission of herbivore-induced

VOCs in exposed corn plants. Although this plant–plant signaling is enhanced if the receiver plant is exposed to ethylene, exposure to ethylene itself is not sufficient to elicit herbivore-induced VOCs in receiver plants (Ruther and Kleier 2005).

Conclusions

It is surprising how little we know about the role of ethylene in plant–herbivore interactions even after half a century of research. Ethylene signaling has been implicated in the elicitation of only eight defense responses (Figure 1). An examination of ethylene's role in plant–herbivore interactions makes it clear that studies which emphasize the role of ethylene in mediating a defense response have failed to analyze its effect on herbivore performance. On the other hand, those studies analyzing the impact of ethylene on an attacking herbivore do not analyze the secondary metabolites that are likely responsible for the changes in herbivore performance. This highlights the value of using species whose plant–herbivore interactions are particularly well studied, especially when ethylene-signaling mutants are incorporated into research programs. The fact that ethylene plays an indirect role may have contributed to the slow rate of progress. For example, the emission of particular VOCs and the accumulation of phenolics and proteinase inhibitor (PI) activity are defense responses that are ethylene mediated (Figure 1), but their elicitation is JA dependent.

A recent study of the regulation of the transcriptome during seedling growth revealed largely non-overlapping responses to seven phytohormones. Genes encoding proteins involved in signaling and transcription were specifically regulated, which is consistent with a role for hormones as triggers of particular signal-transduction cascades (Nemhauser and others 2006). This study contradicts the extensive crosstalk commonly observed among hormones. Kinases and transcription factors are likely important in the integration of signals during the regulation of protective responses against stressors (Fujita and others 2006). In herbivore–plant interactions, ethylene appears to function less as a direct elicitor and more as a modifier of signaling and downstream responses of JA. Whether such interactions are mediated by kinases or transcription factors needs to be determined.

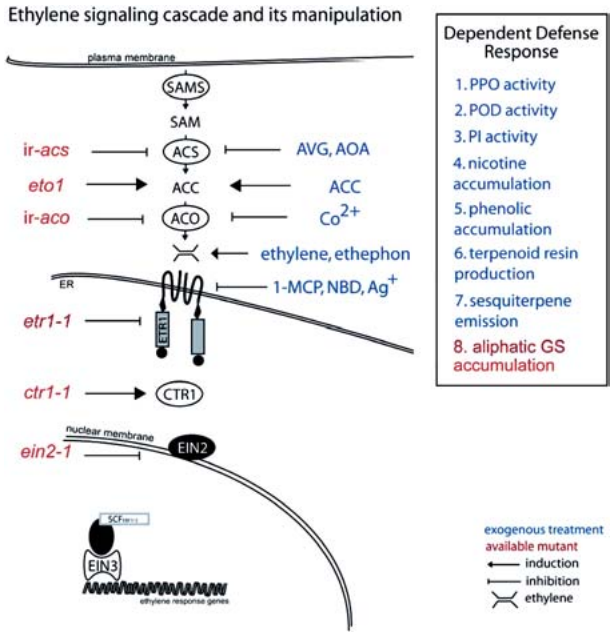

Figure 1. Summary of experimental approaches to manipulate ethylene signaling in plant-herbivore interactions. Plants modulate ethylene responses at the level of synthesis, transport, uptake, and turnover of the hormone itself, or by regulating its perception and signal transduction. Transgenic and mutant plants (marked in red) altered in many of these processes are available but their potential has not been fully utilized in the study of plant-herbivore interactions. Only one ethylene-dependent defense response (aliphatic GS accumulation) has been discovered using mutant plants. The vast majority of research has used exogenous applications of ethylene or its precursors, or various inhibitors (marked in blue), and 7 defense responses are known to be influenced by these treatments. These chemical applications (marked in blue) can have numerous non-specific side effects and their penetration into the different cell compartments is likely limited. In contrast, mutants are available through the *EIN2*-mediated responses at the nuclear membrane. The induced and inhibited steps indicated in the figure refer to the final ethylene response (transcription of ethylene responsive genes) and not necessarily to the activity of the particular step in the pathway. All 8 listed defense responses increase under the presence of ethylene. *Eto1*, *etr1-1*, *ctr1-1*, and *ein2-1* refer to plants mutated in the indicated steps, whereas ir-*acs* and ir-*aco* are inverted-repeat RNAi plants silenced in the indicated steps of the signaling cascade.

Future Directions

We predict that our understanding of ethylene's role in plant–herbivore interactions will be substantially improved once genetic tools (left panel marked red; Figure 1) are incorporated into research programs. Several mutants in ethylene biosynthesis and its downstream signaling cascade are available in *A. thaliana* and *S. lycopersicon* (Czarny and others 2006), species in which both JA-dependent defense responses and ethylene signaling are well defined.

One drawback of genetic manipulations is that when they are driven by nonspecific promoters, they are unable to manipulate ethylene signaling in a tissue-specific manner. Advances in inducible and tissue-specific promoters continue, albeit slowly, but once established, they should be included in the research on ethylene-regulated defenses. A second challenge for genetic manipulation of ethylene biosynthesis is the large gene families of ACC synthases and ACC oxidases, in which specific members mediate specific responses. In the family of tomato ethylene receptors, some members are able to compensate for the loss of other members, which complicates the interpretation of the results (Klee 2004). To avoid these problems, consensus sequences can be used to silence the expression of multiple members in a large gene family by RNAi. In contrast, specific sequence tags, frequently found in the 3'UTR, synthetic miRNAs, or antisense full-length genes, can be used to target the expression of a single family member. Although the results from mutant and transgenic plant analysis must be interpreted cautiously, they will be needed to disentangle the interplay of JA and ethylene in mediating responses to herbivore attack.

When ethylene activity requires the concomitant elicitation of JA, or of any other signal, it is likely to be influenced by numerous other adaptive responses. This might explain why exogenous applications have not been as useful in ethylene research as in the study of JA responses. Recent advances in our understanding of ethylene's role in mediating between-species interactions suggest that it plays an offensive function. The relevance of ethylene emission in competition for light was discovered using ethylene-insensitive *etr1* tobacco plants. That light signals cause the shade avoidance syndrome (SAS) is well known; what is new is that exposure to ethylene is sufficient to elicit some of these responses (Pierik and others 2004). Intraspecific competition, simulated by alterations in red/ far red light ratios, severely reduced the defensive responses of tobacco and tomato plants (Izaguirre and others 2006), suggesting that ethylene helps tailor defense responses in anticipation of competition-mediated stress. Additionally, ethylene may prime a plant, allowing it to respond more quickly to stresses it encounters, as happens when *Pieris rapae* feed on *Arabidopsis* plants that are subsequently inoculated with turnip crinkle virus (TCV). Here, increased sensitivity to virus-induced SA, caused by ethylene, changes the plant's resistance to the pathogen (De Vos and others 2006). Given that performance in a particular habitat requires the subtle tuning of numerous adaptive responses to environmental stimuli, we predict that as genetically modified or mutant plants are more commonly used to explore their performance in natural settings, ethylene's importance as a modulator of ecological responses will be revealed.

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Manuscript III

Tuning the herbivore-induced ethylene burst: the role of transcript accumulation and ethylene perception in *Nicotiana attenuata*

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Frank Kühnemann, Klaus Gase, and Ian T. Baldwin

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This manuscript characterizes the hornworm-induced ethylene burst of *Nicotiana attenuata* plants. Fatty acid-amino acid conjugates (FACs) are identified as oral secretion (OS)-derived elicitors of herbivore-induced ethylene emissions. Transcriptional and feed-back regulation of OS-induced ethylene emissions are described. Floral longevity and OS-induced nicotine accumulation are presented as ethylene-dependent traits relevant for plant-insect interactions.

Under the supervision of Ian T. Baldwin, I planned and performed all experiments including the characterization of the transgenic plants, analyzing the transcript abundance of ethylene biosynthetic genes and their impact on OS-induced ethylene emissions, and describing the ethylene-dependent traits OS-induced nicotine accumulation and flower longevity. The isolation and characterization of all described *NaACS* and *NaACO* genes was performed by Robert A. Winz and Rayko Halitschke. The photoacoustic spectrometer was kindly provided by Frank Kühnemann. Klaus Gase designed and constructed the plasmids used for RNA interference.

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Tuning the herbivore-induced ethylene burst: the role of transcript accumulation and ethylene perception in *Nicotiana attenuata*

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Summary

Caterpillar-induced ethylene emissions play an important role in plant–herbivore interactions. The ethylene burst that ensues after attack exceeds wound-induced ethylene emissions, but the mechanisms responsible remain unknown. Adding larval oral secretions (OS) to wounds mimics this ethylene burst. We demonstrate that fatty acid–amino acid conjugates are the responsible elicitors in *Manduca sexta* OS, and identify genes that are important in OS-elicited ethylene biosynthesis and perception in the larvae's host, *Nicotiana attenuata*, by examining the consequences of gene silencing on OS-elicited ethylene emissions, as quantified by photo-acoustic spectroscopy. OS elicitation increased transcript accumulation of ACC synthase (*ACS*), virus-induced gene silencing of *ACS* halved the OS-elicited ethylene release, and ACC supplementation to ACS-silenced plants restored ethylene emissions, demonstrating that ACS activity limits the rate of release. Silencing three wound- or OS-elicited ACC oxidase (ACO) genes with an *ACO* consensus fragment abolished the OS-elicited ethylene release. Virus-induced gene silencing of each *ACO* individually revealed that only *NaACO2a* and *NaACO3* regulate the OS-elicited ethylene release. Transforming plants with various *etr1-1* constructs rendered them differentially 'deaf' to ethylene, and dramatically increased the OS-elicited ethylene burst, largely without regulating the transcripts of biosynthetic genes. The volume of the OS-elicited ethylene 'scream' was proportional to the plant's deafness, as determined by 1-MCP treatments. We conclude that the OS-elicited ethylene burst is tuned by a tag-team of transcriptional responses and ethylene perception. Ethylene signaling is shown to be essential in regulating two traits that are important in the *N. attenuata–M. sexta* interaction: OS-induced nicotine levels and floral longevity.

Keywords: ethylene biosynthesis, *ACO* transcript accumulation, elicitor, ethylene perception, feedback regulation, induced defense.

Introduction

Ethylene, a simple gaseous hormone, integrates external signals with internal processes, adjusting the plant's phenotype to its environment. The two committed steps of ethylene biosynthesis, the conversion of *S*-adenosyl-L-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC), and its subsequent oxidation to ethylene, are regulated by ACC synthase (ACS) and ACC oxidase (ACO), respectively. ACS and ACO are encoded by multi-gene families consisting of nine and five members, respectively, in *Nicotiana tabacum* (tobacco), and eight and four members, respectively, in *Solanum lycopersicum* (tomato) (Barry *et al.*, 1996; Chen *et al.*, 2003; Nakatsuka *et al.*, 1998). In order to orchestrate complex physiological processes, such as ripening and senescence, in distinct organs, plants control ethylene biosynthesis. They do so by regulating different isoforms of *ACO* and *ACS* in response to particular

developmental and stress cues (Wang *et al.*, 2002). Comparable results have been found for the regulation of wound-elicited ethylene emissions from leaves. The wound-induced transcript accumulation of three members of the *ACO* gene family has been examined in tomato, and only *ACO1* was wound-responsive (Barry *et al.*, 1996). Similar results were found in *Cucumis melo*: both *ACO1* transcripts and activity increased after wounding (Lasserre *et al.*, 1996). *ACO* genes have likewise been shown to be differentially expressed in wounded *Helianthus annuus* seedlings, *Nicotiana glutinosa* leaves and *Vigna radiata* hypocotyls (Kim and Yang, 1994; Kim *et al.*, 1998; Liu *et al.*, 1997).

ACS isoforms are also transcriptionally regulated by wounding. When *Pisum sativum* seedlings were wounded, *Ps-ACS1* did not respond, but *Ps-ACS2* mRNA levels did, peaking after 30 min (Peck and Kende, 1998). Ge *et al.* (2000) identified four wound-induced *ACS* genes in *N. tabacum*, three of which slowly increased within 6 h of wounding. Only the *Nt-ACS4* transcripts accumulated quickly, 1 h after elicitation. Although most ACS isoforms are regulated at the transcript level, additional post-transcriptional regulation is important for the activity of the labile ACS protein. Post-transcriptional regulation could be inferred in *eto1* and *eto3* ethylene-overproducing mutants because their levels of *ACS* mRNA are the same as those of wild-type (WT) seedlings (Woeste *et al.*, 1999). Similarly, over-expressing and silencing *ACS* in tobacco plants did not influence ethylene biosynthesis (Knoester *et al.*, 1997). The pioneering work of S. Zhang's group demonstrated conclusively that ACS activity is regulated by mitogen-activated protein kinases (MAPKs), which in turn have been shown to be wound-regulated (Kim *et al.*, 2003; Liu and Zhang, 2004; Zhang and Klessig, 1998).

Although ethylene perception has been assumed to play a central role in both positive and negative feedback regulation of ethylene biosynthetic enzymes (Yang and Hoffman, 1984), this assumption has not been rigorously tested. Ethylene is perceived through a family of six receptor genes in tomato (*LeETR1-6*) with structural similarities to the five receptor genes in Arabidopsis (*ETR1*, *ERS2*, *ERS1*, *ETR2* and *EIN4*), which have been shown to be negative regulators of ethylene responses (Hua and Meyerowitz, 1998). Ethylene receptors are homologous to His kinases and can be structurally separated into the sensor, kinase and receiver domains (Chang *et al.*, 1993). Ethylene perception can regulate ethylene signaling in at least two ways. First, a plant's sensitivity to ethylene can be decreased by increasing receptor expression. Once a receptor has bound ethylene, it can no longer repress ethylene-inducible genes. Thus, synthesis of new receptors probably terminates transitory ethylene responses by decreasing ethylene sensitivity below a threshold necessary for the response, as proposed by Klee (2004). In tomato, *LeETR1* and *LeETR2* are constitutively expressed, but the transcripts of the other four receptor genes are highly regulated. For example, *LeETR4* and *NR* (*LeETR3*) mRNA abundance respond strongly to *Xanthomonas campestris* infection (Ciardi *et al.*, 2000).

A second way that ethylene perception can regulate wound-induced ethylene biosynthesis is by feedback regulation of *ACS* and *ACO* gene expression. All studies addressing this question have used pharmacological approaches rather than ethylene perception mutants. A seminal study with mung bean hypocotyls inferred feedback regulation of the wound-induced *ACO* mRNA accumulation by applying ethylene with and without either 2,5-norbornadiene (NBD), an ethylene action inhibitor, or aminooxyacetic acid (AOA), an inhibitor of ACS activity. Each inhibitor was sufficient to repress wound-induced *ACO* mRNA abundance, while the simultaneous addition of ethylene restored the accumulation of *ACO* mRNA (Kim and Yang, 1994).

Abiotic stresses, such as wind, frost, exposure to heavy metals and drought frequently result in wounding, and wound-induced ethylene production has been thoroughly studied (Abeles *et al.*, 1992). Plants are wounded by both biotic and abiotic factors, and if a plant is to adjust its phenotype in an adaptive manner, it must be able to distinguish between the various environmental factors that cause wounding. Herbivore-attacked plants are known to reconfigure their wound response when herbivore-specific elicitors in insect oral secretions and regurgitants (OS) are introduced into wounds during feeding. Known elicitors in the OS of lepidopteran larvae include fatty acid–amino acid conjugates (FACs), which regulate oxylipin signaling and the regulated defense responses in maize and *N. attenuata* (Alborn *et al.*, 1997; Halitschke *et al.*, 2001). *Vigna unguiculata* and *Phaseolus vulgaris* inceptins, herbivore-specific fragments of the plant's ATP synthase, are also OS-derived elicitors responsible for eliciting specific defense responses including jasmonic acid (JA) and ethylene bursts (Schmelz *et al.*, 2006).

The ethylene produced in response to herbivore attack dramatically exceeds that produced by a similar amount of mechanical wounding (reviewed by von Dahl and Baldwin, 2007). Herbivore-induced ethylene is known to modify the accumulation of direct defenses, including phenolics (Hudgins and Franceschi, 2004), alkaloids (Kahl *et al.*, 2000) and protein-based defenses (Harfouche *et al.*, 2006; O'Donnell *et al.*, 1996), as well as the release of volatile organic compounds thought to function as indirect defenses (Horiuchi *et al.*, 2001; Schmelz *et al.*, 2003). Rather than being the principal elicitor of herbivore-induced defense responses, ethylene appears to play a more subtle role modulating other defense signals, including JA (Schmelz *et al.*, 2003; and reviewed by von Dahl and Baldwin, 2007).

Although the herbivore-induced ethylene burst has been known about for more than 50 years, little is known about its regulation. A recent transcriptional study of four distinct *ACS* gene family members in *Picea sitchensis* after weevil

attack and mechanical wounding revealed strong increases in *ACS2* and *ACS3*, moderate increases in *ACO* transcripts, and no change in the remaining two *ACS* genes (Ralph *et al.*, 2007). When *Nicotiana attenuata* plants are attacked by *Manduca sexta* larvae, the plants release significant quantities of ethylene, a response that can be mimicked by applying *M. sexta* OS to puncture wounds (Kahl *et al.*, 2000). Here we used reverse genetic approaches to understand the regulation of the OS-elicited ethylene burst in *N. attenuata* plants. We characterized the OS-elicited accumulation patterns of transcripts from genes important in ethylene biosynthesis and perception, silenced the expression of these genes by stable transformation and virus-induced gene silencing (VIGS), and examined the consequences of the gene silencing on OS-elicited ethylene emissions, as measured by photo-acoustic spectroscopy, and for two phenotypes important for the *M. sexta*–*N. attenuata* interaction: nicotine production and flower longevity.

Results

FACs are elicitors of the OS-induced ethylene burst

The *M. sexta*-specific elicitation of ethylene emissions is clearly distinguishable from the wound-induced ethylene response in *N. attenuata* plants (Kahl *et al.*, 2000). As in these earlier experiments, applying water to puncture wounds in excised leaves elicited only a weak increase in ethylene emissions, whereas applying *M. sexta* OS elicited a nearly fivefold increase (Table 1; ANOVA, $F_{5,19} = 64.507$, $P < 0.001$). We tested the two most abundant FACs at the concentrations found in *M. sexta* OS for their ability to elicit ethylene emissions. Treatment with FACs increased ethylene emissions to similar levels as seen after OS treatment. In contrast, neither the detergent-containing buffer nor FAC-free ion-exchanged OS showed any ethylene-inducing activity (Table 1).

Table 1 FACs are sufficient to induce OS-dependent ethylene emissions in *N. attenuata*

Treatment	Ethylene emission (nl g^{-1} FM h^{-1})
Control	1.14 ± 0.09^a
Water	1.99 ± 0.26^a
OS	9.72 ± 1.15^b
iexOS	1.82 ± 0.18^a
FACs	8.56 ± 1.17^b
Triton	1.45 ± 0.14^a

Mean ± SE ($n = 4$) ethylene emissions of excised leaves as determined by 'stop-flow' measurements after wounding and immediate application of water, *M. sexta* oral secretions (OS), ion-exchanged OS (iexOS), the two most abundant fatty acid–amino acid conjugates (FACs) or 0.025% Triton to the puncture wounds. Control leaves were unwounded and left without treatment. One of two replicated experiments with equivalent results is shown. Superscript letters indicate significant differences between the treatments (Bonferroni-corrected *post hoc* test, $P < 0.05$).

N. attenuata ACS *and* ACO *gene family members*

N. attenuata shoot and root cDNA libraries were screened for *ACS* and *ACO* genes using probes generated from known *N. tabacum* sequences. Sequencing the isolated phage clones identified four different *ACS* (*NaACS1*, *NaACS2*, *NaACS3a* and *NaACS3b*) and *ACO* (*NaACO1*, *NaACO2a*, *NaACO2b*, and *NaACO3*) genes in *N. attenuata*, with high (70–95%) sequence identity to corresponding genes of *N. tabacum*, *N. glutinosa* and *Solanum lycopersicon* (Supplementary Table S1). Specific probes hybridizing to the 3′ end of each cDNA were generated, and were verified by slot-blot analysis demonstrating specificity above a minimal level of background cross-reactivity (data not shown). Southern blot analysis of *N. attenuata* genomic DNA revealed single copies of all characterized *ACS* and *ACO* genes with the exception of *NaACS1*, which is likely to have a second copy (Supplementary Figure S1).

Transcript accumulation of all characterized genes was analyzed in hydroponically grown plants by Northern blot analysis. No transcripts of *NaACO2b*, *NaACS2* or *NaACS3b* were detected in young or old roots, stem tissues, rosette or stem leaves, flower buds or flowers (data not shown). *NaACO1* and *NaACO3* showed weak constitutive transcript abundance in leaves of untreated control plants; similar results were found for *NaACO2a* and *NaACS3a* in root tissues (Supplementary Figure S2). Transcript levels of all *NaACO* genes increased after 3 h in response to *M. sexta* feeding: *NaACO1* showing the strongest response (Supplementary Figure S2), while *NaACS1* transcripts showed only a very weak *M. sexta*-elicited response (Supplementary Figure S2).

OS-induced ethylene emissions require NaACS3a *and are limited by ACS activity*

The weak *M. sexta*-induced transcriptional regulation of *ACS* genes (Supplementary Figure S2) and the results of several other studies suggest that post-transcriptional regulation is involved in controlling ethylene emissions. We used VIGS to rapidly determine the relevance of *NaACS* transcript abundance in the OS-elicited ethylene emissions from *N. attenuata*. Silencing *NaACS* using a pTVacs VIGS construct suppressed the OS-induced release of ethylene by 50% in comparison to that for plants inoculated with the empty vector (EV) (Figure 1a; ANOVA, $F_{3,13} = 33.346$, $P < 0.0001$). OS-elicited *NaACS3a* transcript accumulation was reduced to 25% of that in EV plants (Figure 1, inset). No differences in wound-induced ethylene emissions between EV and acs

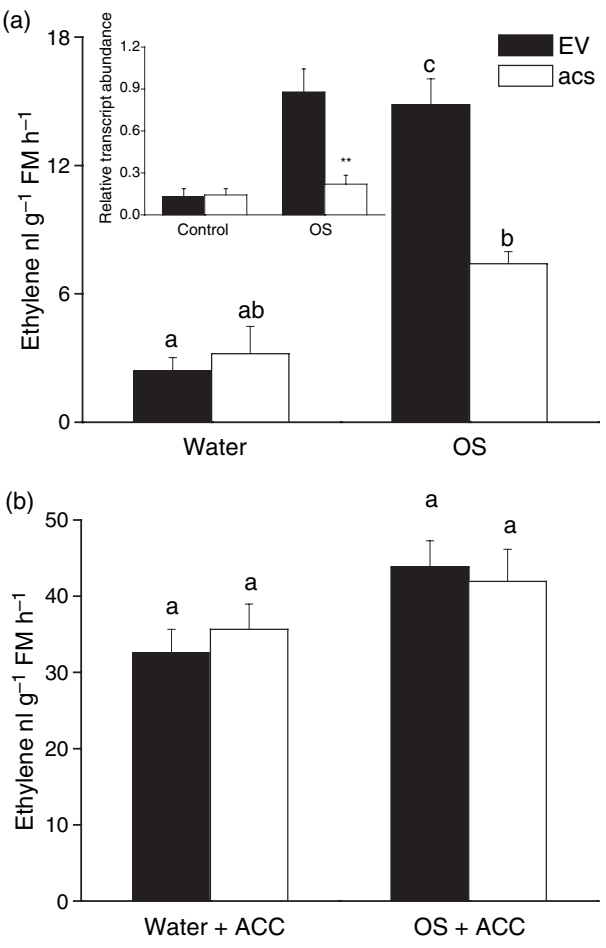

Figure 1. NaACS activity is the rate-limiting step of OS-induced ethylene emissions.
Nicotiana attenuata plants were inoculated with a pTVacs vector (acs, open bars) or an empty vector (EV, filled bars) construct to control for VIGS-related phenotypes. Ethylene emissions (mean and SE, $n = 3$) were measured by 'stop-flow' measurements of single leaves after wounding and treatment of the wounds immediately with *Manduca sexta* oral secretions (OS) or water (water) (a), or both treatments were supplemented with ACC to a final concentration of 5 mM (b). Different letters indicate significant differences between the treatments (Bonferroni-corrected *post hoc* tests, $P < 0.05$). Inset: the relative transcript abundance (mean and SE, $n = 5$) of *NaACS3a* was analyzed by RT-PCR 1 h after OS elicitation in control acs and EV plants. Transcript levels were normalized to those of an endogenous control gene (*ECI*) (Student's *t* test, $^{**}P < 0.001$).

plants were observed. OS-elicited ethylene emissions of EV plants exceeded the OS-elicited ethylene emissions of WT plants (Table 1, Figures 1–3), suggesting that the VIGS procedure interfered with ethylene biosynthesis.

ACS activity is often regarded as the rate-limiting step in ethylene biosynthesis. ACS activity can be crudely mimicked by adding ACC to wounded tissues. Adding ACC to wounds strongly increased ethylene emissions of OS-elicited leaves (Figure 1). Furthermore, no differences in ethylene emissions between EV and acs plants were observed in leaves supplemented with ACC (Figure 1b; ANOVA, $F_{1,9} = 0.031$, $P = 0.8656$). Thus, ACO activity is sufficient to convert the product of ACS, ACC, into ethylene, and the supply of ACC is likely to be the rate-limiting step in OS-elicited emissions.

The role of NaACO2a *and* NaACO3

The apparent surfeit of ACO activity in the presence of unlimited ACC was surprising in light of the strong *M. sexta*-elicited increase in *ACO* transcript accumulation (Supplementary Figure S2). We used two approaches to determine the relevance of the elicited changes in *ACO* transcript abundance: silencing all *ACO* genes by stable transformation and transiently silencing individual *ACO* gene family members by VIGS to further estimate their relevance to OS-elicited ethylene emissions.

A consensus sequence for a region with high homology in all three *ACO* genes (*NaACO1*, *NaACO2a* and *NaACO3*) was used to create an inverted-repeat (ir) construct to comprehensively silence the OS-responsive *ACO* gene expression. A single-insertion ir-*aco* line was characterized (Supplementary Figure S3) and bred to homozygosity. ir-*aco* plants did not differ from WT plants in growth morphology or in above- and below-ground biomass (data not shown). Silencing *ACO* expression was analyzed by quantitative RT-PCR. The transcript accumulation of all three *ACO* genes was significantly reduced in ir-*aco* plants compared to WT plants (Figure 2a), and the OS-induced ethylene emissions of ir-*aco* plants (1.14 ± 0.09 nl g^{-1} FM h^{-1}) were lower than those of untreated WT plants (Figure 2b).

Using gene-specific VIGS constructs, we transiently silenced individual *ACO* genes to examine their role in OS-induced ethylene release. Transcript analysis of VIGS plants revealed that individual *ACO* genes were highly and specifically silenced. Levels of *NaACO1* (ANOVA, $F_{3,39} = 10.666$, $P < 0.0001$), *NaACO2a* ($F_{3,35} = 4.5$, $P = 0.009$) and *NaACO3* ($F_{3,39} = 13.556$, $P < 0.0001$) were significantly reduced in plants inoculated with the corresponding gene-specific VIGS construct without influencing transcript accumulations of the non-target *ACO* genes and *NaACS3a* (Figure 3a, *P* values >0.05).

OS-induced ethylene emissions were significantly reduced in plants inoculated with pTVaco2 (aco2) and pTVaco3 (aco3) constructs in comparison to EV plants (ANOVA, $F_{3,44} = 9.824$, $P \leq 0.0008$) but only marginally in pTVaco1-inoculated plants (ANOVA, $F_{3,44} = 9.824$, $P = 0.1128$) (Figure 3b). Thus, silencing the transcript accumulation of *NaACO2a* and *NaACO3* significantly reduced OS-induced ethylene emissions, while silencing the transcript accumulation of *NaACO1* was only marginally effective.

Individual ACS *and* ACO *genes are differentially regulated*

The VIGS analysis demonstrated that transcript levels of *NaACS3a* and particular *NaACO* genes are crucial for

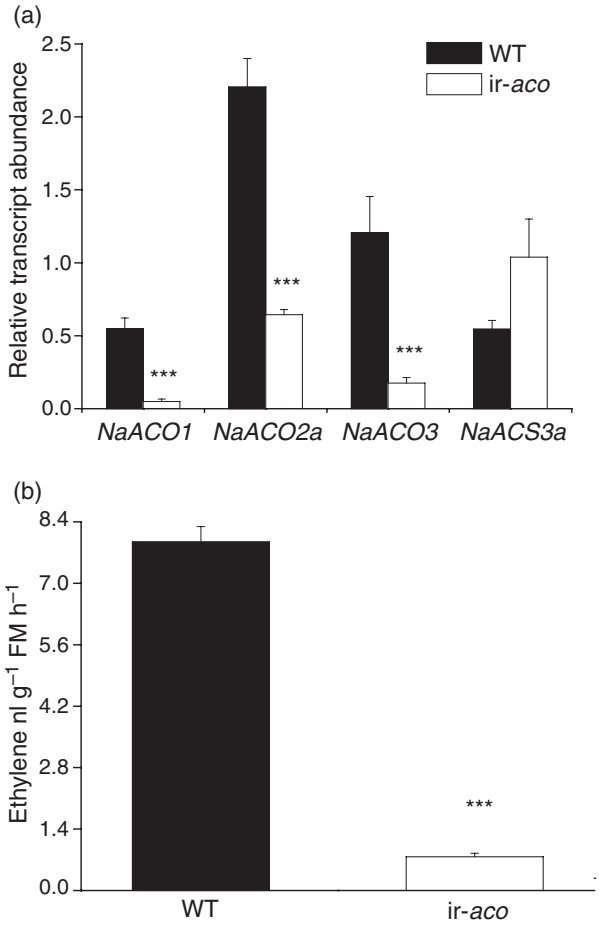

Figure 2. OS-induced transcript accumulation and ethylene emissions are silenced in ir-*aco* plants.
(a) The relative transcript abundance (mean and SE, $n = 5$) of *ACO* and *ACS* genes was analyzed by RT-PCR in ir-*aco* (open bars) and WT (filled bars) *N. attenuata* plants. *NaACS3a* and *NaACO2* transcript levels were quantified 1 h after OS elicitation, and those of *NaACO1* and *NaACO3* were quantified 3 h after OS elicitation. Transcript accumulations were normalized to those of an endogenous control gene (*ECI*) (students *t* test, $^{***}P < 0.0001$).
(b) Ethylene emissions (mean and SE, $n = 3$) were determined by 'stop-flow' measurements of two leaves after wounding and OS elicitation (Student's *t* test, $^{***}P < 0.0001$).

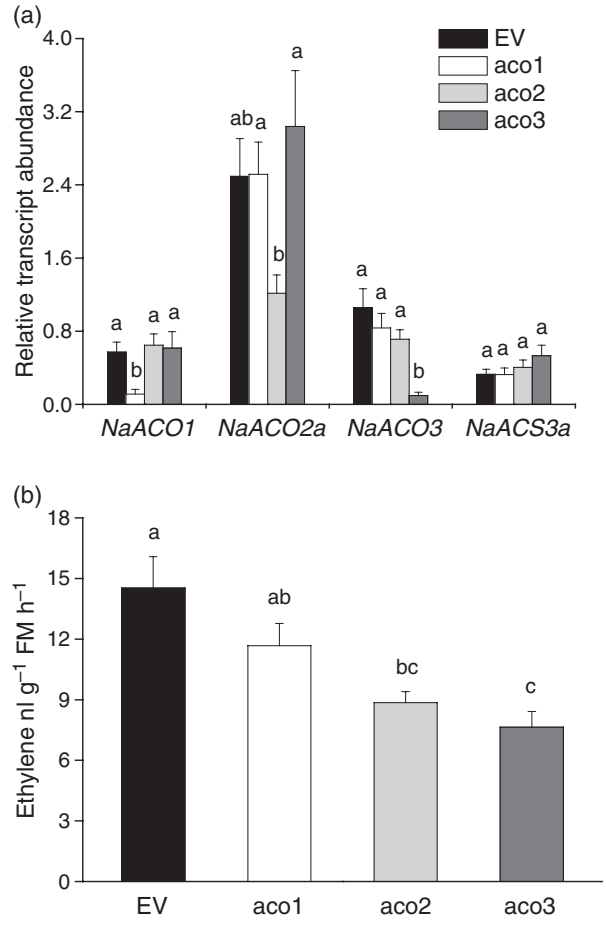

Figure 3. Dependence of herbivore-induced ethylene emissions on *NaACO* gene expression.
Nicotiana attenuata plants were transiently silenced in their accumulation of *NaACO1*, *NaACO2a* or *NaACO3* transcripts by inoculation with the following vectors: pTVaco1 (aco1; white bars), pTVaco2 (aco2; gray bars) and pTVaco3 (aco3; dark gray bars). Empty vector constructs (EV; black bars) were inoculated to control for VIGS-related phenotypes.
(a) OS-induced relative transcript abundance was analyzed by RT-PCR 1 and 3 h after *M. sexta* OS elicitation for *NaACS3a* and *NaACO2a* and for *NaACO1* and *NaACO3*, respectively. Values were normalized to those of an endogenous control gene (*ECI*).
(b) Ethylene emissions were measured by 'stop-flow' measurements of single leaves after wounding and OS elicitation.
Mean values are shown, error bars represent SE ($n = 12$), and different letters indicate significant differences as determined by ANOVA (Bonferroni-corrected *post hoc* tests, $P < 0.05$).

determining OS-elicited ethylene emissions. To investigate their OS-dependent regulation, a quantitative RT-PCR analysis was performed. *NaACS3a*, *NaACO1*, *NaACO2a* and *NaACO3* transcripts responded to OS elicitation, and could be categorized as either late- or early-responding genes (Figure 4). The early-responding genes, *NaACS3a* and *NaACO2a*, displayed maximum transcript levels within the first hour of elicitation, and both increased after OS application to wounds. Transcript levels of *NaACO2a* declined more slowly than did those of *NaACS3a*, and reached the levels of controls much later, around 6 h after elicitation (Figure 4). The late-responding genes, *NaACO1* and *NaACO3*, reached maximum transcript levels between 2 and 4 h after elicitation but differed strongly in their responses to the two treatments. *NaACO1* transcripts were hardly detectable in control plants; *NaACO3* transcripts were constitutively abundant. Whereas *NaACO1* transcript levels were elicited by wounding and amplified by OS elicitation, *NaACO3* transcript levels were responsive to wounding but wound-induced transcript accumulation was not further increased by OS (Figure 4).

Having determined that *ACS* and *ACO* genes responded very differently to OS elicitation, we were interested in the

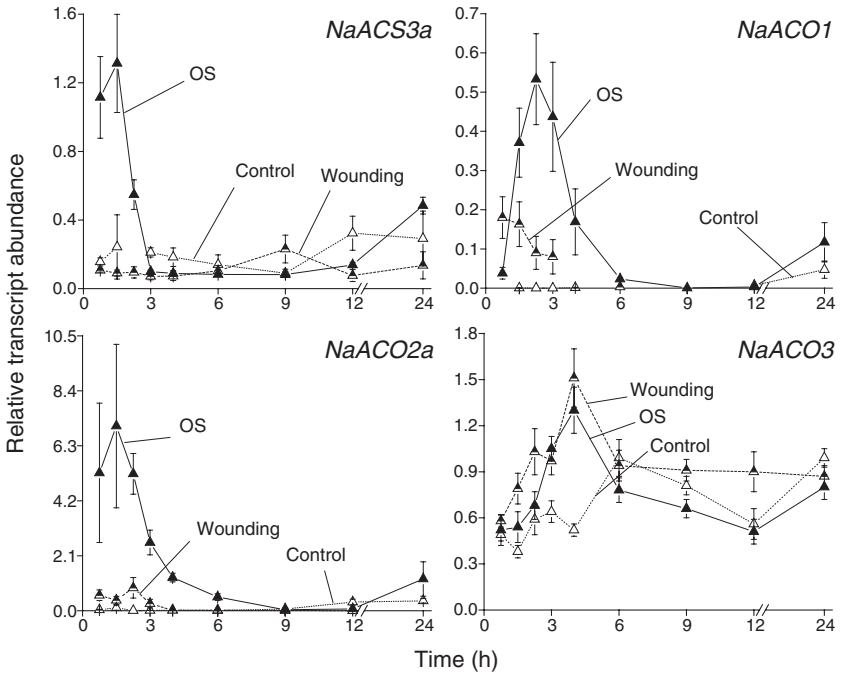

Figure 4. Levels of OS- and wound-regulated transcripts of genes responsible for ethylene biosynthesis.
Relative transcript abundance ± SE of *NaACS3a*, *NaACO1*, *NaACO2a* and *NaACO3* as analyzed by RT-PCR. Transcript levels of the individual genes were normalized to the transcript abundance of an endogenous control gene (*ECI*). The leaves of five individual plants were harvested at the indicated time points after elicitation by mechanical wounding and the application of either water (wounding, half-filled symbols, dashed line) or *M. sexta* larvae OS (filled symbols, solid line). Control plants were not wounded (open symbols, dotted line). Where data points are not shown, the transcript levels were undetectable.

influence of ACC, an integral part of the ethylene biosynthetic pathway, on the OS-elicited transcriptional responses. ACC supplementation changed the pattern of OS-elicited transcript levels. ACC supplementation to wounded EV plants decreased the levels of *NaACS3a* and *NaACO2a* compared to OS-elicited EV plants (Supplementary Figure S4), while levels of *NaACO1* and *NaACO3* transcript accumulation were not influenced (Supplementary Figure S4).

Ethylene perception negatively regulates OS-induced ethylene emissions with only marginal changes in transcript levels

To determine whether ethylene perception regulates the release of OS-induced ethylene, we quantified transcript levels of the ethylene receptor gene *NaETR1* of *N. attenuata* after wounding and OS elicitation, and found that they were not regulated by either treatment (Figure 5a, inset). To determine whether perceived ethylene regulates ethylene biosynthesis, we used both transformed plants and a pharmacological approach to silence ethylene perception. In order to obtain at least one ethylene-'deaf' line, we generated several ethylene-insensitive *N. attenuata* plants with varying abilities to sense ethylene by heterologously expressing the mutant Arabidopsis receptor *etr1-1* using three different pRESC2ETR constructs. We generated homozygous lines harboring single insertions for 35s-etr1a and 35s-etr1c and a line with two transgene insertions for 35s-etr1b (Supplementary Figure S3). All three lines accumulated transgene transcripts to varying degrees as determined by Northern blotting (Supplementary Figure S5). Plants expressing the three different constructs displayed different growth responses in the triple response assay. Root growth in 35s-etr1c, but not 35s-etr1a and 35s-etr1b, plants was reduced in response to ACC, while hypocotyl elongation was insensitive to ACC application in plants of all three lines (Supplementary Figure S6).

The release of OS-elicited ethylene from ethylene-insensitive transformants was much stronger than that from WT plants. We measured the OS-induced ethylene release of whole plants of all four genotypes continuously and non-destructively over a 15 h period (Figure 5a). Higher maximum levels as well as prolonged emission of ethylene were observed for ethylene-insensitive plants compared to WT plants. 35s-etr1a plants released only slightly more ethylene than did WT plants, whereas 35s-etr1b and 35s-etr1c plants showed strong and intermediate amplifications of OS-elicited ethylene emissions, respectively.

Increased OS-elicited levels of *NaACS3a* transcripts in ir-*aco* plants compared to WT plants (Figure 2a; Student's *t* test, $P = 0.06$) suggested that ethylene perception regulated the negative feedback of *NaACS3a* transcripts. We further analyzed the OS-induced transcript accumulation of *ACO* and *ACS* in ethylene-insensitive plants. No significant differences were observed at their maxima 1 and 3 h after elicitation for the early-responding and late-responding genes, respectively (Supplementary Figure S7A). We prolonged the observation to 12 h, and, with the exception of a slightly extended accumulation of *NaACS3a* and *NaACO2a* at 3 h in 35s-etr1b plants (Supplementary

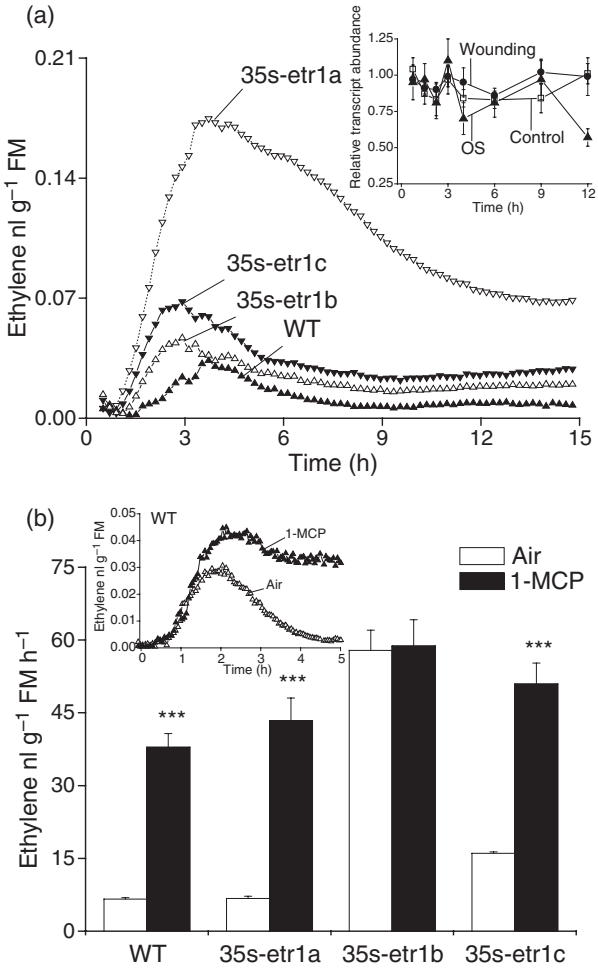

Figure 5. Ethylene perception negatively regulates OS-elicited ethylene emissions.

(a) Online ethylene measurement of whole plants after OS elicitation of three leaves. WT (filled upward-pointing triangle), 35s-etr1a (open upward-pointing triangle), 35s-etr1b (open downward-pointing triangle) and 35s-etr1c (filled downward-pointing triangle) plants were induced and measured over 15 h. Data from one experiment (of three replicated experiments) are shown. Inset: the relative transcript abundance ± SE of *NaETR1* as analyzed by RT-PCR. Transcript accumulation was normalized to the accumulation of an endogenous control gene (*ECI*). Leaves of five individual plants were harvested at the indicated time points after wounding (filled circles) or OS elicitation (filled triangles). Control plants were not wounded (open squares).

(b) Ethylene emissions (mean + SE) were analyzed by 'stop-flow' measurements of single leaves of four individual plants from each of the WT and 35s-etr1a–c genotypes after OS elicitation. Ethylene accumulated for 10 h. Prior to OS treatment, six plants were enclosed in 20 l containers with (filled bars) and without 1-MCP (open bars) overnight, to block all ethylene receptors (Student's *t* test, ***$P < 0.001$). Inset: ethylene emissions from WT plants were measured online using a laser-acoustic spectrometer. Three leaves were wounded and elicited with OS at the onset of measurement. Ethylene receptors were blocked by an 8 h exposure to 1-MCP (filled symbols) during the previous night. Control plants were OS-elicited without prior exposure to 1-MCP (air, open symbols).

Figure S7B; ANOVA, *NaACS3a* $F_{3,8} = 11.012$, $P = 0.0047$; *NaACO2a* $F_{3,11} = 10.596$, $P = 0.0014$), no differences were detected.

A similar pattern of amplified and extended ethylene emissions was observed in WT plants rendered ethylene-insensitive by 1-MCP exposure (Figure 5b, inset). We further quantified the ethylene released during the first 10 h after OS elicitation in the ethylene-insensitive *etr* lines with and without prior overnight exposure to 1-MCP (Figure 5b). 1-MCP treatment resulted in a fourfold increase in the OS-induced ethylene emissions of WT plants. A similar MCP-dependent amplification was observed in 35s-etr1a plants (fourfold) and 35s-etr1c plants (threefold). No significant increase was observed in the elevated ethylene emissions of 35s-etr1b plants in response to MCP treatment. Interestingly, the difference in the OS-elicited ethylene emission before and after 1-MCP treatment of the genotype (Figure 5b) was correlated with the OS-elicited ethylene emissions of the genotype (Figure 5a), which is consistent with the hypothesis that the 35s-etr1 transformants differed quantitatively in their ability to perceive ethylene, and, when rendered completely 'deaf' to ethylene by the 1-MCP treatment, all genotypes released similar quantities of ethylene. These results establish a strong association between ethylene perception and ethylene emission, or in other words, ethylene-'deaf' plants become ethylene 'screamers', and the volume to which they modulate their ethylene scream is proportional to their degree of deafness.

Ethylene perception mediates phenotypes relevant for plant–insect interactions

Finally, we characterized two traits relevant to plant–insect interactions in transgenic plants retarded in ethylene signaling: OS-induced nicotine accumulation and floral longevity. JA-dependent nicotine accumulation is OS-amplified by 48 and 4% in WT plants (Figure 6a). The observed inducibility of WT nicotine levels varied strongly, but lay within the range of observed nicotine induction for *N. attenuata*. Nicotine levels depend not only on the age of the plants and the elicitor used, but also on the growth environment conditions. The OS-dependent amplification was always stronger (55–100%) in the ethylene-insensitive 35s-etr1b and 35s-etr1c plants and in ir-*aco* plants (Figure 6a). Furthermore, the lifetimes of flowers were significantly prolonged in all transgenic plants compared to WT plants. The 'deafness' of the various ethylene mutants correlated with the length of time that flowers remained turgid and attached to the plant: 30% of WT flowers were still open and turgid after the third night (Figure 6b), but 56 and 80% of the flowers of 35s-etr1a and 35s-etr1c plants, respectively, and 100% of all the flowers on 35s-etr1b plants were still turgid and attached after the third night. ir-*aco* plants had 20% more open flowers in the third night than WT plants (Figure 6b).

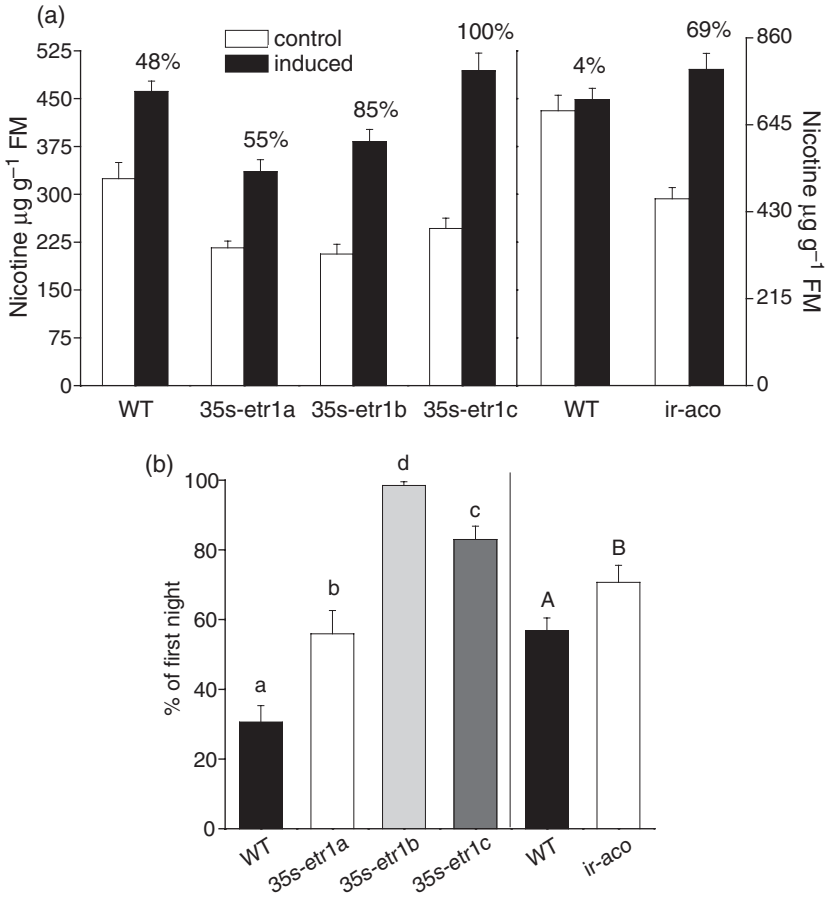

Figure 6. Ethylene signaling regulates defense and floral traits in *N. attenuata*.
(a) Nicotine levels (mean and SE, $n = 8$) in OS-elicited (filled bars) and control (open bars) plants 4 days after wounding and OS elicitation. Data from two independent experiments are presented; the experiments compare WT, 35s-etr1a, 35s-etr1b and 35s-etr1c plants, and also WT and ir-*aco* plants. The percentage increase in the constitutive level measured in elicited plants is given for each genotype.
(b) Percentage (mean and SE) of flowers remaining open and turgid on the third night of two independent experiments comparing WT, 35s-etr1a, 35s-etr1b and 35s-etr1c plants, and WT and ir-*aco* plants. The maximum longevity of WT *N. attenuata* flowers is three nights. Different letters of the same case indicate significant differences between genotypes as determined by ANOVA (Bonferroni-corrected *post hoc*, $P < 0.05$).

Discussion

The roles of NaACS3a, NaACO1, NaACO2a *and* NaACO3 *in OS-elicited ethylene production*

When *M. sexta* OS are applied to puncture wounds in *N. attenuata* leaves, ethylene emissions increase fivefold in comparison to those of plants in which leaf wounds are treated with water (Kahl *et al.*, 2000). We cloned four members of the *ACS* and *ACO* gene families that were abundant in a cDNA library of *M. sexta*-attacked *N. attenuata* leaves. Transcripts of *ACS* genes were hardly detectable: *NaACS1* was weakly expressed in leaves, *NaACS3a* was constitutively expressed in roots. In contrast, transcripts of three *ACO* genes (*NaACO1*, *NaACO2a* and *NaACO3*) were abundant in leaves attacked by *M. sexta* larvae as analyzed by Northern blotting (Supplementary Figure S2). A more detailed RT-PCR analysis revealed *NaACO1* to be a late-responding gene that was amplified by OS; *NaACS3a* and *NaACO2a* are early-responding genes with transcript levels that increased within 1 h of elicitation and were only regulated by OS, and *NaACO3* is a late-responding and wound-responsive gene (Figure 4). The relative transcript abundance of the specific genes varied between the Northern blot and the data obtained by RT-PCR, which might have been caused by differences in the elicitation treatments: *M. sexta* feeding versus a single OS elicitation event (Supplementary Figures S2 and 4). The data represent relative transcript levels, and between-gene comparisons should be made cautiously. Herbivore-induced transcript regulation has been studied in Sitka spruce, where transcript accumulation of two *ACS* genes and one *ACO* gene has been observed in response to weevil attack and mechanical wounding. Whereas both *ACS* genes were mostly wound-responsive within the first 6 h after elicitation, transcript levels were up-regulated 48 h after attack by white pine weevil (*Pissodes strobi*) to an extent that exceeded levels in mechanically wounded plants (Ralph *et al.*, 2007).

To determine the relevance of each of the *ACS* and *ACO* genes to the OS-elicited ethylene release in *N. attenuata*, we silenced the expression of each gene and quantified the OS-elicited ethylene burst. Using VIGS on *NaACS3a* reduced OS-elicited ethylene emissions to 50% of these of EV-inoculated plants (Figure 1a). Adding ACC to wounds increased ethylene emissions threefold and obliterated differences in OS-elicited EV and *NaACS3a*-silenced plants. These results are consistent with the substantial literature demonstrating that ACS activity, by supplying ACC substrate for the abundant ACO

activity, is often the rate-limiting step in elicited ethylene production (Yang and Hoffman, 1984).

As all three *ACO* gene family members were strongly regulated by wounding and OS, we silenced their expression simultaneously by transforming plants with an *ACO* consensus sequence in an inverted repeat (ir) orientation. OS-induced ethylene biosynthesis in ir-*aco* plants was profoundly reduced; after OS elicitation, plants only released ethylene in amounts equal to those of untreated WT leaves (compare Figure 2b and Table 1). These results are consistent with results obtained by the antisense expression of pTOM13 (ACO1) in tomato, which reduced transcript accumulation and protein activity and severely reduced ethylene emissions in wounded leaf discs (Hamilton *et al.*, 1990). To determine which of the three ACOs were decisive, we used VIGS to silence each gene individually, and found OS-elicited ethylene production in *NaACO2a*- and *NaACO3*-silenced lines to be significantly reduced (Figure 3a).

The fact that silencing *NaACO1* had no effect on OS-elicited ethylene emissions, while silencing *NaACO3* had the strongest effect, was surprising (Figure 3a). Unlike the transcript accumulation of NaACO3 that was elicited by wounding alone, the transcripts of *NaACO1* responded specifically to OS elicitation (Figure 4). The fact that both *NaACO1* and *NaACO3* respond slowly suggests that these genes may be more important in maintaining the ethylene release during sustained herbivore attack, rather than in initiating the OS-elicited ethylene burst. The transcript levels of plants silenced for *NaACS3a*, *NaACO1*, *NaACO2a* and *NaACO3* were reduced to 25, 25, 50 and 10% of those of OS-elicited EV plants, respectively (Figure 1a, inset, and Figure 3b), and insufficient gene silencing is unlikely to explain the large amounts of ethylene emitted by plants silenced by VIGS of their *aco1* transcripts. Nevertheless, the low overall abundance of *NaACO1* might explain its negligible contribution to the OS-elicited ethylene burst. Whether its contribution becomes more important during continuous herbivore attack, as Northern blot analysis indicates, needs to be investigated further (Supplementary Figure S2). Given that OS-elicited ethylene production levels of EV plants were on average 50% higher than those of WT plants (compare Figures 1 and 3 with Table 1), and that only in the stably transformed ir-*aco* lines was the OS-elicited ethylene burst completely abolished (Figure 2), it is possible that additional genes responsible for ethylene biosynthesis are activated in OS-elicited VIGS plants. Virus infection of *N. glutinosa* elicited the accumulation of a different set of *ACO* genes than was observed during the wound response (Kim *et al.*, 1998), and hence developing stably transformed ir-*aco1* lines may be necessary to finally determine the role of ACO1 in mediating the ethylene burst. Increased enzyme activity of ACO is due to the *de novo* synthesis of proteins in tomato, and the actual increase in transcript levels is related to the accumulation of active enzymes (Barry *et al.*, 1996). These findings in tomato are likely to apply to *Nicotiana* species also, as the transcriptional activation of *ACO* genes is mediated by similar developmental signals and *cis*-acting sequences in tomato and *N. plumbaginifolia* (Blume and Grierson, 1997).

Adding ACC to OS inhibited transcript accumulation of the early-responding genes, *NaACS3a* and *NaACO2a*, but had no effect on the late-responding genes, *NaACO1* and *NaACO3*, in EV plants (Supplementary Figure S4). This indicates either that the extraordinarily high amounts of ethylene (Figure 1b) inhibited transcript accumulation, as previously shown (Nakatsuka *et al.*, 1998), or that ACC directly inhibited transcript accumulation in cross-talk between ACS and ACO. Interestingly, OS elicitation increased *NaACS3a* transcripts in ir-*aco* plants (Figure 2b); such an increase could be explained by either increased ACC accumulation in ir-*aco* plants, which are clearly unable to oxidize ACC to ethylene, or by the inhibition of *NaACS3a* through feedback based on the perception of insufficient quantities of ethylene. As these plants do not produce OS-elicited ethylene, their inability to produce ethylene in sufficient quantities may cause them to increase the expression of *NaACS3a*, the apparent rate-limiting step. Because the results from the ACC supplementation experiments contradict the first hypothesis, we were interested in how plants that are unable to perceive their own ethylene emissions regulate their OS-elicited ethylene biosynthesis.

Ethylene perception regulates herbivore-induced ethylene production

We cloned a fragment of *NaETR1* and found that transcript levels of this ethylene receptor gene did not change in response to OS or wounding (Figure 5a, inset). ETR1 is commonly constitutively expressed, and its transcripts are not regulated during fruit ripening or in response to pathogen attack in tomato (Klee, 2004). Plants are able to fine-tune their responses to environmental stimuli by regulating their perception of ethylene. In tomato, mRNA levels of some of the ethylene receptors responded to pathogen inoculation and ethylene exposure, which suggests that regulation of these genes may serve to additionally tune ethylene signaling (Ciardi *et al.*, 2000).

In order to generate ethylene-insensitive plants, we heterologously expressed the *etr1-1* mutant ethylene receptor from Arabidopsis in *N. attenuata*, a method that has been successfully used in many species to silence ethylene perception and has helped to elucidate the role of ethylene signaling in several processes (Knoester *et al.*, 1998). We used three different constructs and obtained three ethylene-insensitive transgenic lines, which we called 35s-etr1a, 35s-etr1b and 35s-etr1c. These lines displayed varying degrees of impaired ethylene signaling, which were observed during a triple response assay (Supplementary Figure S6), and in

the amplification of OS-elicited ethylene emissions after 1-MCP exposure (Figure 5b). These observations suggest that a plant's degree of ethylene sensitivity (its 'deafness') determines the amount of ethylene produced in response to OS elicitation. *N. attenuata* plants expressing the mutant *etr1-1* receptor emitted more OS-induced ethylene than did WT plants (Figure 5a). Although the OS-elicited ethylene emission of WT, 35s-etr1a and 35s-etr1c plants increased after blocking the remaining active receptors by 1-MCP exposure, this was not the case for 35s-etr1b plants (Figure 5b). We propose that negative feedback regulation of ethylene biosynthesis (i.e. ethylene-deaf plants becoming ethylene 'screamers') is mediated by ethylene perception. This phenomenon was observed after the pollination of petunia flowers over-expressing the mutant Arabidopsis ethylene receptor *etr1-1* (Wilkinson *et al.*, 1997). Several studies have used ethylene exposure and inhibited ethylene perception by 1-MCP or NBD treatments to investigate the positive and negative feedback regulation of ethylene production (proposed by Yang and Hoffman, 1984). In some studies, ethylene exposure is apparently autocatalytic for wound-induced ethylene production, such as in *N. glutinosa*, *Cucumis melo* and *Vignia radiata* (Kim and Yang, 1994; Kim *et al.*, 1998; Lasserre *et al.*, 1996). In contrast, exposure to ethylene inhibited its own biosynthesis by decreasing ACC synthase transcript levels in wounded pea seedlings (Peck and Kende, 1998), and *Spodoptera*-induced ethylene production was inhibited by 1-MCP treatment in corn leaves (Schmelz *et al.*, 2003). In contrast, OS-elicited ethylene emissions from 35s-etr1 plants were significantly higher than those from WT plants (Figure 5a).

Interestingly, the increased OS-elicited ethylene emissions of genetically rendered ethylene-deaf plants was not accompanied by increases in *ACS* and *ACO* transcripts at the times of their maximum abundance; however, the OS-elicited ethylene releases of 35s-etr1b plants were accompanied by increased transcript levels of the early genes, *NaACS3a* and *NaACO2a*, 3 h after elicitation (Supplementary Figure S7). Thus, the waxing and waning of herbivore-induced ethylene emissions include the regulation of *ACS* and *ACO* transcripts. The disconnect between the marginal increases in *ACS* and *ACO* transcripts in ethylene-insensitive plants and their dramatic ethylene emissions suggests that post-transcriptional regulation is involved in the down-regulation of the OS-elicited ethylene burst in WT *N. attenuata* plants. ACS phosphorylation by salicylic acid-induced protein kinase (SIPK) has been demonstrated to be a key step in ethylene biosynthesis in Arabidopsis (Kim *et al.*, 2003; Liu and Zhang, 2004), and SIPK activity is strongly increased by OS elicitation (Wu *et al.*, 2007). Furthermore, *NaACS3a* is most likely a homolog of *AtACS6*, which has been shown to be phosphorylated by MPK6 at a site in its extended C-terminus (Liu and Zhang, 2004). However, a second explanation might be the regulation of other *ACS* and *ACO* transcripts that were not detected when the library was screened for enriched herbivore-induced genes. For example, sequencing the Arabidopsis genome revealed about a dozen members of the *ACS* gene family, and the genome of *N. attenuata* is likely to comprise more than the four *ACS* genes described (Arabidopsis Genome Initiative, 2000). The differential expression and negative feedback regulation of ethylene biosynthesis have been observed previously in the development and ripening of tomato fruits (Nakatsuka *et al.*, 1998). Clearly biochemical characterizations of ACS and ACO protein abundance in response to OS elicitation are required to verify these hypotheses.

We propose the following model for OS-elicited ethylene production in *N. attenuata* (Figure 7). The introduction of FACs into plant wounds during larval feeding is sufficient to elicit OS-induced ethylene emissions, which are partially regulated by *NaACS3a*, *NaACO2a* and *NaACO3* transcript levels. Transcripts of the early genes, *NaACS3a* and *NaACO2a*, accumulate within the first hour, during initiation of the OS-elicited ethylene burst, but are down-regulated in the presence of high levels of ACC, which might suppress gene abundance after its conversion to ethylene. At this early stage of the induction of ethylene biosynthesis, 1 h after elicitation, the lack of ethylene production increases *NaACS3a* transcript accumulation. A tag-team of OS-elicited ethylene biosynthetic transcripts becomes active when the late-responding genes, *NaACO1* and specifically *NaACO3*, accumulate; these genes are not responsive to the addition of ACC. Termination of the OS-elicited ethylene response involves the perception of ethylene, which mediates the down-regulation of *ACS* and *ACO* transcripts as well as of *ACS* and *ACO* enzymes.

Ethylene perception strongly regulates phenotypes Important in plant–insect interactions

Ethylene plays a subtle role in plant–insect interactions, frequently modulating the response of other active signal compounds or hormones (von Dahl and Baldwin, 2007). For example, the nicotine accumulation of *N. attenuata* plants in response to *M. sexta* herbivory, OS application and MeJA induction has been shown to be attenuated by ethylene (Kahl *et al.*, 2000). During the elicitation of this potent direct defense, levels of JA-induced putrescine *N*-methyltransferase (*PMT*) are inhibited by the plant's ethylene production (Winz and Baldwin, 2001). OS elicitation increased nicotine levels in WT *N. attenuata* plants by 4% and 48%, but in the mutants impaired in ethylene signaling, this increase was amplified to 55–100% (Figure 6a). The extent of the OS-elicited nicotine increase correlated with the lack of ethylene signaling. This cross-talk between JA and ethylene signaling in response to *M. sexta* attack probably provides a significant fitness benefit to *N. attenuata* plants growing under

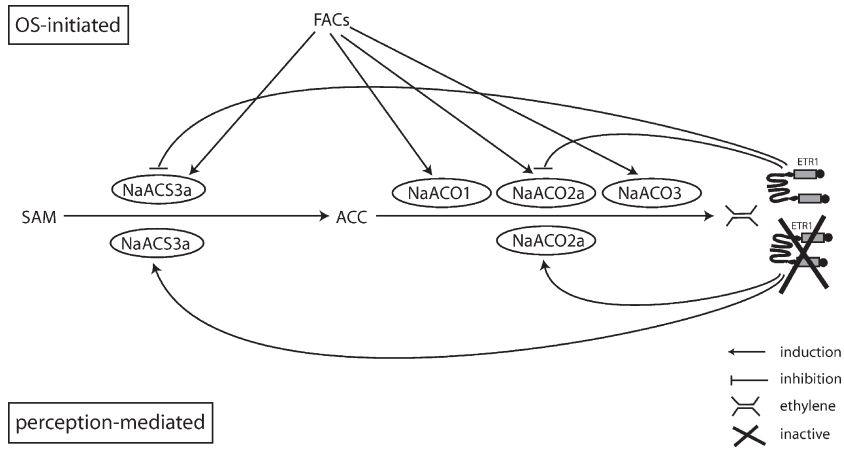

Figure 7. Model of OS-elicited ethylene biosynthesis in *N. attenuata* leaves.
OS-regulated ethylene biosynthesis. When *M. sexta* larvae attack and feed on plants, fatty acid–amino acid conjugates (FACs) are introduced into wounds. Treating wounds with the two most abundant FACs is sufficient to elicit ethylene emissions similar to those elicited when wounds are treated with OS. Within the first hour, OS elicitation strongly increases *NaACS3a* and *NaACO2a* transcript levels; transcript levels of the late-responding genes (*NaACO1* and *NaACO3*) reach their maximum around 3 h. Whereas *NaACO1* responds only to OS elicitation, *NaACO3* is wound-regulated. Adding ACC suppresses the OS-elicited transcript accumulation of the early genes, *NaACS3a* and *NaACO2a*, which might be mediated by increased ethylene production, as ACO activity is sufficient to oxidize both OS-elicited ACC production as well as exogenously added ACC. The lack of ethylene production during the initiation of ethylene biosynthesis increases OS-induced *NaACS3a* transcript levels in ir-*aco* plants that are silenced in *ACO* transcript accumulation.
Regulation mediated by perception. Although *NaETR1* transcripts are unchanged by wounding or OS elicitation, plants expressing the mutant receptor increase OS-induced ethylene emissions in proportion to their inability to perceive ethylene. The increased production of ethylene in ethylene-'deaf' plants is partially explained by the increased transcript abundance of *NaACS3a* and *NaACO2a*.

intense intra-specific competition. The ethylene burst elicited by *M. sexta* attack does not occur when plants are attacked by *Tupiocoris notatus*, *Spodoptera exigua* or *Myzus persicae nicotianae* (C. Diezel, C.C. von Dahl, and I.T. Baldwin, unpublished results), and allows plants to down-regulate a costly but less effective defense against the nicotine-adapted herbivore *M. sexta* (Voelckel *et al.*, 2001).

Floral longevity is defined as the period of time from anthesis to floral senescence. Longevity enhances the floral display and plays an important role in the reproductive ecology of plants by influencing the total number of pollinator visits and increasing the quantity of a plant's progeny (Ashman and Schoen, 1996). At the same time, floral displays may incur fitness costs by prolonging resource allocation to the reproductive tissue and extending appearance to herbivores. *N. attenuata* plants with experimentally elevated emissions of benzyl acetone, the plant's most abundant floral fragrance, were browsed more frequently than were control plants, resulting in fewer capsules being produced (Baldwin *et al.*, 1997). Average flower lifetime was extended up to threefold on ethylene-insensitive *N. attenuat*a plants. 35s-etr1b plants, which displayed the strongest ethylene-insensitive phenotype, produced 'permaflowers' that retained white and turgid corollas long after pollination and well into the seed-filling stage of capsule development. That ir-aco plants displayed the weakest increase in floral longevity might be related to the fact that other *ACO* genes are not silenced by the consensus region of the herbivore-responsive *ACO* genes that we used to design the construct. Senescence and abscission of flowers determines flower lifetime, and these are known to be regulated by ethylene (Patterson and Bleecker, 2004).

Our results emphasize that, although ethylene plays a secondary rather than a primary role in the elicitation of plant responses in several plant–insect interactions, ethylene-'deaf' plants are likely to suffer severe fitness costs as a result of their inability to rapidly adjust their phenotypes to the dynamics of their abiotic and biotic environments. We propose that ethylene plays a central role in adaptive phenotypic plasticity.

Experimental procedures

Plant and insect growth

Seeds of an inbred line of *Nicotiana attenuata* Torr. ex. Wats. (synonymous with *Nicotiana torreyana* Nelson and Macbr.) and of the transformed lines were germinated on Gamborg B5 media as described by Krügel *et al.* (2002). Seedlings were transplanted into potting soil after 10 days, and grown for an additional 2–3 weeks. Hydroponic plants were grown as described by van Dam *et al.* (2001) in 1 l containers. Hydroponic solutions were supplemented with 2 mmol KNO_3 the day before experimental treatments commenced. All plants were grown under high-pressure sodium lamps (800–1000 μmol m^{-2} sec^{-1} photosynthetically active radiation) with a 16 h/8 h light/dark cycle, at 28°C and 65% humidity during the light cycle. Experiments were conducted with late rosette-stage plants or shortly after bolting and before flower production. *Manduca sexta* larvae were hatched from eggs (Carolina Biological Supply, http://www2.carolina.com) and reared on *N. attenuata* foliage.

Elicitation treatments

The response to herbivory was analyzed in *N. attenuata* plants damaged by actively feeding *M. sexta* larvae. Three 3rd–5th instar larvae were allowed to feed on the plants, resulting in the removal of approximately half the shoot mass. *M. sexta* attack was simulated by creating puncture wounds with a fabric pattern wheel and immediately applying either water or *M. sexta* larvae OS to the puncture wounds. These procedures, referred to as wound and OS elicitation, respectively, allow wound-induced and herbivore-specific responses to be differentiated, and the precise timing of the elicitation to be determined. *M. sexta* larvae OS were diluted 1:1 (v/v) with water, and 20 μl were applied to puncture wounds created by a pattern wheel to the leaf lamina; 20 μl water were applied in the wound elicitation treatments. Controls were not elicited. To identify the elicitor in *M. sexta* larval OS that is responsible for the induction of ethylene release, additional solutions were applied to the puncture wounds: 0.06 mM *N*-linolenoyl-L-Gln and 0.17 mM *N*-linolenoyl-L-Glu in 0.05% Triton-X100 (Sigma; http://www.sigmaaldrich.com/), ion-exchanged OS eluted four times through ion-exchange columns containing 400 mg of the basic ion-exchange resin Amberlite IRA-400 (Sigma), or 0.05% Triton X100. All test solutions were diluted 1:1 (v/v) with water, and 20 μl were applied to puncture wounds created by a pattern wheel on the leaf surface. 1-aminocyclopropane-1-caboxylic acid (ACC; Sigma) was added to water or OS to a final concentration of 5 mM in the 1:1 diluted solutions. In a preliminary experiment, we determined that using 0.05% Triton, 0.05 M MES buffer or water as solvents did not affect the related ACC-induced ethylene emissions (data not shown).

Plants were exposed to 1-MCP for 8 h during the dark cycle. According to the method described by Kahl *et al.* (2000), 500 mg of Ethylblock (0.43% 1-MCP; van der Sprong, http://www.sprongla.nl) were placed in a vial. After adding 10 ml of an alkaline solution (0.75% KOH + NaOH) to release 1-MCP, the vial was immediately enclosed in a 20 l container populated with six plants scheduled to receive 1-MCP exposure. Control plants were enclosed in containers without 1-MCP.

Isolating N. attenuata ACS, ACO *and* ETR1 *genes*

cDNA libraries prepared from shoot and root material of *M. sexta*-damaged *N. attenuata* plants (Hermsmeier *et al.* 2001) were screened with *ACS*- and *ACO*-specific probes (Supplementary Table S2) according to the manufacturer's protocols (λZAPII library; Stratagene; http://www.stratagene.com/). *ACS* probes radioactively labeled with Rediprime II (Amersham Pharmacia; http://www5.amershambiosciences.com/) were generated by PCR using pBluescript SK- plasmids (Stratagene) containing the *Nicotiana tabacum NtACS1* (Q07262) and *NtACS2* (CAA67118) cDNAs as a template. Similarly, a PCR probe for *ACO* was generated from *NtACO1* (CAA67119, CAA82646). Hybridized blots were washed four times at 65°C in 2x SSC, 0.1% SDS before autoradiography. Initial positive-phage clones were isolated, PCR-screened for full-length cDNA inserts, and sequenced on an ABI310 sequencer using the Big Dye terminator kit (Applied Biosystems; http://www.appliedbiosystems.com/).

NaETR1 was cloned by PCR using genomic DNA as a template and the following primers: ETR1–1F (5′-GGTGCTTTCATAGTTCTTTGTGG-3′) and ETR1-1R (5′-AGCCTTGAAAGATCCAAGACATC-3′). Products were gel-purified, cloned into the pCR® 2.1 TOPO vector (Invitrogen; http://www.invitrogen.com/), and sequenced.

Nucleic acid analysis

Plant genomic DNA was isolated by the cetyltrimethylammonium bromide method as described by Winz and Baldwin (2001). DNA samples (10 μg) were restriction-digested, size-fractionated by 0.8% agarose gel electrophoresis, and Southern-blotted onto nylon membrane (NEN-DuPont , http://www.bioscience.org/company/dupont.htm). Northern blot analysis was performed as described by Winz and Baldwin (2001). Gene-specific probes were generated by PCR (Supplementary Table S2). Purified PCR fragments were labeled with ^{32}P using a random prime labeling kit (Rediprime II; Amersham Pharmacia). Probe specificity was verified by slot-blotting (Hoefer, http://www.hoeferinc.com/) a dilution series (200, 20 and 2 ng) of *NaACS* or *NaACO* plasmids onto a nylon membrane as described in the manufacturer's protocol (GeneScreen*Plus*; NEN-DuPont) and hybridized with the various *ACS* or *ACO* gene-specific ^{32}P-labelled PCR fragments. Membranes were subject to analysis by autoradiography or a phosphoimager (FLA 3000; Fujifilm Europe http://fujifilm.de/).

Additionally, real-time PCR assays were used to quantify transcript accumulation. The youngest fully expanded leaves of rosette-stage plants were elicited as described above. Treated leaves of five replicate plants were harvested at different time points (45, 60, 90 and 135 min, and 3, 4, 6, 9, 12 and 24 h) after elicitation. Total RNA was extracted using TRI reagent (Sigma), and cDNA was synthesized from 20 ng of total RNA according to the manufacturer's protocol (Applied Biosystems). Real-time PCR was performed on an SDS7700 (Applied Biosystems) using the qPCR™ reagent kit (Eurogentec, http://www.eurogentec.com/code/en/hp/asp). Primer and fluorescence dye-labeled probe combinations for the individual assays are listed in Supplementary Table S2. The expression of target genes was normalized to the expression of the endogenous control gene *ECI* (sulfite reductase) as described by Bubner *et al.* (2004).

Generation of transgenic plants

For the construction of pRESC5ACO1 (11.0 kb) carrying an *NaACO1* fragment in an inverted repeat orientation under the control of the CaMV 35S promoter, the 0.2 kb *Nco*I–*Pst*I and 0.2 kb *Sac*I–*Xho*I fragments of plant transformation vector pRESC501 (10.9 kb; Bubner *et al.*, 2006) were replaced by two identical, divergently oriented 0.2 kb PCR fragments of the *NaACO1* gene (for primer sequences see Supplementary Table S3). To generate plasmid pRESC2ETR1 (13.4 kb) carrying a mutated *etr-1* gene in a sense orientation under the control of the CaMV 35S promoter, the mutated chromosomal region of the *Arabidopsis thaliana etr1* gene (AC020665, positions 33 116–40 381, mutation G to A at 36538), resident on a plasmid, was cut with *Aat*II, blunt-ended with T4 DNA polymerase, and digested with *Nco*I. The 3.7 kb fragment thus obtained was ligated to the 9.7 kb fragment of pRESC201 (Bubner *et al.*, 2006) and treated with *Xho*I, T4 DNA polymerase and *Nco*I. For sense expression of the *etr1-1* gene under the control of the CaMV 35S promoter, the mutated cDNA of the *Arabidopsis thaliana etr1* gene (positions 147–2382 of NM_105305, mutations G to A at 359 and C to A at 600) was obtained as 3.0 kb *Eco*RV–*Pst*I fragment from plasmid pCD-2, and cloned into pRESC2GER (Lou and Baldwin, 2006) before being cut with *Pst*I and *Sma*I (9.7 kb). Unnecessary DNA between *etr1-1* and the downstream CaMV terminator was removed from the resulting plasmid pRESC2ETR2 (12.7 kb) by *Pst*I and partial *Bam*HI digestion, blunting the ends and recirculating the 12.1 kb fragment, to obtain pRESC2ETR3.

The *Agrobacterium tumefaciens* (strain LBA 4404)-mediated transformation procedure was used as described by Krügel *et al.* (2002) using pRESC5ACO1, pRESC2ETR1, pRESC2ETR2 and pRESC2ETR3. Successful transformants and their homozygous progeny were selected by hygromycin-resistance screening. The number of transgene insertions was determined by Southern blot analysis and hybridization with a probe specific for the hygromycin resistance gene *hptII* (Supplementary Table S2). Ethylene-insensitive *etr* lines were characterized using the triple response assay (Guzmán and Ecker, 1990). Seeds were germinated on 40 ml GB5 media with and without 20 μg 1-aminocyclopropane-1-caboxylic acid (ACC; Sigma). After 3 days, when radicals emerged, lights were turned off. Seedlings were grown in the dark for 2 days, after which root and hypocotyl length were measured.

Virus-induced gene silencing

To transiently silence ethylene biosynthetic genes, we used a virus-induced gene silencing (VIGS) system based on the tobacco rattle virus (Ratcliff *et al.*, 2001) optimized for *N. attenuata* genes (Saedler and Baldwin, 2004). Constructs for transient transformation (pTV-ACS3a, pTVACO1, pTVACO2, and pTVACO3) were designed by cloning the fragments (obtained using the respective primers listed in Supplementary Table S3) into the binary pTV00 vector. *Agrobacterium* strain GV3101 carrying the respective constructs was co-inoculated with the helper vector pBINTRA6 into young *N. attenuata* plants as described by Saedler and Baldwin (2004). Plants were incubated in darkness for 2 days afterwards, and experiments were performed 14–20 days after inoculation. To monitor the progress of VIGS, we silenced phytoene desaturase (PDS), which results in the visible bleaching of green tissues. Only leaves of the same age as the bleached leaves on *pds*-VIGS plants were used in the analysis.

Ethylene measurements

Ethylene emissions were measured continuously and non-invasively in real-time with a photo-acoustic spectrometer (INVIVO, University of Florida, Gainesville). The light source consisted of a line-tunable CO_2 laser, and the detection device was a resonant photo-acoustic cell. The 'fingerprint' spectrum of ethylene in the infra-red spectral region allows for a highly sensitive analysis by alternating measurements of the photo-acoustic signal on the CO_2 laser lines 10p14 and 10p16. The detection device consisted of two acoustic cells. One cell was filled with a known ethylene concentration (516 ppb) from a calibration gas reservoir, which was used to calibrate and continuously adjust the laser line. The sampling cell was calibrated with the gas (516 ppb) before the start of each experiment. To remove hydrocarbons, air was cleaned by oxidizing all organics: air was passed through a platinum catalyst at 540°C (Sylatech, http://www.sylatech.de/) before being directed to the sampling devices. For 'stop-flow' measurements, the youngest fully expanded leaves of rosette-stage plants were elicited and detached at the petiole. Excised leaves were transferred to 250 ml cuvettes, and ethylene was allowed to accumulate in the headspace for 5 h or otherwise indicated. The cuvettes were flushed with a flow of purified air at 130–150 ml min^{-1}, which had also passed through a cooling trap to remove CO_2 and H_2O. For continuous measurements, individual soil-grown plants were placed in 3 l glass cuvettes, and the three youngest, fully expanded leaves were elicited with OS or wounding. Cuvettes were exposed to a controlled flow of catalytically cleaned air (30 ml min^{-1}). Four airtight cuvettes were sampled sequentially with a ten-port sampling valve (Knauer http://www.knauer.net/d/d_index.html). The air in the sampling chambers was equilibrated using small fans and subsequently flushed through cooling traps to remove CO_2 and H_2O. During the measurement, air was sampled sequentially from each cuvette. One data point consists of 40 measurements per cuvette and cycle (about 120 sec).

Nicotine and floral longevity

Treated leaves of OS-elicited plants were harvested 4 days after induction. Leaves of the same age were harvested from undamaged plants as controls. Approximately 100 mg leaf samples were flash-frozen in liquid nitrogen and stored at −80°C for analysis of secondary metabolites by HPLC as described by Keinänen *et al.* (2001).

Flower lifetimes were determined by labeling the flowers after their first night with fully opened corollas. Open, white and turgid flowers still attached to the plants were counted each morning within the first 2 h of the light cycle.

Statistical analysis

The data were analyzed by ANOVA with Bonferroni corrected *post hoc* tests or by Student's *t* test. Data were log-transformed to meet the assumptions of equal variances of the statistical tests.

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Supplementary Material

The following supplementary material is available for this article online:

Table S1. *ACS* and *ACO* gene families are highly conserved in Solanaceous plants.

Table S2. Primer selection for gene-specific probes of *NaACS*, *NaACo*, and *ERTI*.

Table S3. Primer selection of plasmid design for transformation.

Figure S1. Southern blot analysis of *N. attenuata ACS* and *ACO* genes.

Figure S2. *M. sexta*-induced transcription accumulation of *ACO* and *ACS* genes.

Figure S3. Number of transgene insertions of ir-*aco* and 35s-etr lines.

Figure S4. *ACC*-induced transcript accumulation in EV inoculated *N. attenuata plants.*

Figure S5. Endogenous and heterologous expression of *ETR1* and 35s-etrl lines.

Figure S6. Triple responses assay of *N. attenuate etr*-lines.

Figure S7. Transcript levels of ethylene biosynthesis genes are not altered in ethylene insensitive plants.

This material is available as part of the online article from http://www.blackwell-synergy.com

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Manuscript IV

Sebacina vermifera promotes the growth and fitness of *Nicotiana attenuata* by inhibiting ethylene signaling

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This manuscript describes the growth-promoting effect of association with *Sebacina vermifera* in *Nicotiana attenuata* in relation to jasmonate signaling and the regulation of its costly defense response, trypsin proteinase inhibitor (TPI). Furthermore, using seedling assays and transgenic plants, we analyzed *S. vermifera's* growth-promoting effects and their dependence on the plant's ethylene signaling.

Oz Barazani performed the majority of the presented experiments under the supervision of Ian T. Baldwin. I assisted with the triple response assay and the ethylene measurements, and determined the transcript abundance of ethylene biosynthesis genes using RT-PCR. I helped edit the manuscript.

Sebacina vermifera Promotes the Growth and Fitness of *Nicotiana attenuata* by Inhibiting Ethylene Signaling[1][W]

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Sebacina vermifera, a growth-promoting endophytic fungus, significantly increases *Nicotiana attenuata*'s growth but impairs both its herbivore resistance and its accumulation of the costly, jasmonic acid (JA)-regulated defense protein, trypsin proteinase inhibitor (TPI). To determine if the fungi's growth-promoting effects can be attributed to lower TPI-related defense costs, we inoculated transformed *N. attenuata* plants silenced in their ability to synthesize JA, JA-isoleucine, and TPI by antisense (lipoxygenase 3 [as-*lox3*] and Thr deaminase [as-*td*]) and inverted repeat (ir-*tpi*) expression, and found that inoculation promoted plant growth as in untransformed wild-type plants. Moreover, herbivore-elicited increases in JA and JA-isoleucine concentrations did not differ between inoculated and uninoculated wild-type plants. However, inoculation significantly reduced the morphological effect of 1-aminocyclopropane-1-carboxylic acid on wild-type seedlings in a triple response assay, suggesting that ethylene signaling was impaired. Furthermore, *S. vermifera* failed to promote the growth of *N. attenuata* plants transformed to silence ethylene production (1-aminocyclopropane-1-carboxylic acid oxidase [ir-*aco*]). Inoculating wild-type plants with *S. vermifera* decreased the ethylene burst elicited by applying *Manduca sexta* oral secretions to mechanical wounds. Accordingly, oral secretion-elicited transcript levels of the ethylene synthesis genes *NaACS3*, *NaACO1*, and *NaACO3* in inoculated plants were significantly lower compared to these levels in uninoculated wild-type plants. Inoculation accelerated germination in wild-type seeds; however, uninoculated wild-type seeds germinated as rapidly as inoculated seeds in the presence of the ethylene scrubber $KMnO_4$. In contrast, neither inoculation nor $KMnO_4$ exposure influenced the germination of ir-*aco* seeds. We conclude that *S. vermifera* increases plant growth by impairing ethylene production independently of JA signaling and TPI production.

Plants that associate with beneficial rhizosphere microorganisms, which include symbiotic and other endophytic and free-living rhizobacteria, often grow better than plants that don't (Glick, 1995; Varma et al., 1999; Strack et al., 2003; Barazani et al., 2005; Waller et al., 2005). The symbiotic associations of plants with arbuscular mycorrhizae (AM), ectomycorrhizal fungi, and nitrogen-fixing bacteria are referred to as mutualistic interactions. Symbiotic fungi or bacteria benefit from the plants' carbohydrates, while plants benefit when the supply of more stationary nutrients such as nitrogen, phosphorus, calcium, magnesium, zinc, copper, and iron is increased. The sequence of events that leads to the development of symbiotic association involves regulating defense-related genes, which have been characterized during the early establishment of AM symbiosis (Kapulnik et al., 1996; Garcia-Garrido and Ocampo, 2002; Liu et al., 2003; Balestrini and Lanfranco, 2006). In addition, phytohormones, usually associated with plants' responses to biotic stresses, were shown to play a role in mycorrhizal development. In *Allium sativum*, for example, treatment with jasmonic acid (JA) was shown to stimulate mycorrhizal development (Regvar et al., 1996), and JA and its conjugated form JA-Ile accumulated in the roots of barley (*Hordeum vulgare*) colonized with *Glomus intraradices* (Hause et al., 2002). Furthermore, silencing the allene oxide cyclase gene, which encodes allene oxide cyclase, an enzyme of the JA biosynthesis pathway, suppressed AM colonization (Isayenkov et al., 2005), suggesting that jasmonates are associated with establishment of a strong carbon sink in the roots (Hause et al., 2002; Strack et al., 2003). In contrast, the mycorrhization of tobacco (*Nicotiana tabacum*) with *G. mosseae* reduced salicylic acid (SA) levels in the plant, and colonization by the fungus was suppressed by constitutive SA synthesis (Medina et al., 2003).

In addition to establishing symbiotic associations, plants are associated with a diverse range of free-living microorganisms that increase plant performance (Glick, 1995). This group of nonspecific plant growth-promoting rhizobacteria, whose members can grow inside the root or on its surface, are known to increase plant fitness by secreting iron scavenging siderophores, reducing nitrates, fixing nitrogen, and producing plant growth regulators (Glick, 1995; Somers et al., 2004). In addition to supplying growth-limiting resources, plant-microbe interactions are often associated with

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increased resistance to plant pathogens (Pieterse et al., 2000; Borowicz, 2001; Pozo et al., 2004). Recently, Waller et al. (2005) related the increase in grain yield and resistance among the pathogenic fungi of barley inoculated with *Piriformospora indica* to modifications in the antioxidative status of the plant (Waller et al., 2005). *P. indica*, a beneficial endophytic fungus (Sebacinales), was first isolated in India from the rhizospheres of *Prospis juliflora* and *Zizyphus nummularia* (Verma et al., 1998). *P. indica* was shown to increase the survival of regenerated tobacco plantlets (Sahay and Varma, 1999) and to increase the root and shoot biomass of *Zea mays*, tobacco, and *Petroselinum crispum* (Varma et al., 1999), as well as of *Spilanthes calva* and *Withania somnifera* (Rai et al., 2001).

Recently, we reported that *P. indica* and its genetically related species *Sebacina vermifera* increase the growth and fitness of *Nicotiana attenuata* (Barazani et al., 2005). However, the increased performance of inoculated *N. attenuata* came at the expense of the plant's resistance to attack from the larvae of one of the plant's most important lepidopteran insect herbivores, *Manduca sexta*. The decrease in herbivore resistance could be attributed to the down-regulation of trypsin protein inhibitor (TPI) activity (Barazani et al., 2005). Plants recognize that the specialist *M. sexta* is attacking when they are wounded and elicitors present in the larvae's oral secretions (OS) are introduced into the wounds during feeding (Halitschke et al., 2001). Applying OS to wounds is sufficient to induce a burst of two phytohormones, ethylene and JA, which activate a wide array of genes responsible for direct and indirect defenses, including the gene responsible for the accumulation of TPI. Consequently, the specialist larvae grow more slowly, presumably because the protein digestion in their gut is inhibited (Zavala et al., 2004b). However, the resistance benefits of TPI expression come at a substantial fitness cost for the plant. *N. attenuata* plants expressing TPIs produce 20% fewer seeds than do isogenic plants transformed to silenced TPI production; restoring TPI production by transforming an ecotype of *N. attenuata* naturally deficient in TPI production reduces lifetime seed production by 20% (Zavala et al., 2004a). Hence we hypothesized that the increase in growth and seed production that *N. attenuata* realizes from associating with *S. vermifera* results from the down-regulation of TPI production (Barazani et al., 2005).

Here we falsify this hypothesis with plants transformed in their ability to produce TPIs and in two steps in the JA signaling cascade required to elicit TPI production and demonstrate that *S. vermifera*'s growth-promoting effects result from alterations in ethylene signaling. We show that: (1) increases in plant performance related to the fungus are independent of JA and TPI, but depend on the ability of the plant to produce ethylene; (2) the beneficial effects of *S. vermifera* on seed germination and seedling growth are ethylene dependent; and (3) the OS-induced ethylene emission and increased transcript accumulation of ethylene biosynthesis genes are reduced in *S. vermifera*-inoculated plants compared to uninoculated plants.

RESULTS

TPI Activity and Transcript Accumulation Is Suppressed in OS-Elicited Inoculated Plants

TPI activity in OS-elicited rosette-stage leaves, 72 h after OS elicitation was nearly twice as high in uninoculated plants compared to *S. vermifera*-inoculated *N. attenuata* wild-type plants (Fig. 1). This significant (*t* test, $F_{1,6} = 6.67$; $P = 0.04$) difference in defense metabolite deployment was also detectable at the transcriptional level 6 h after elicitation (Fig. 1, inset). In response to OS elicitation, TPI transcripts accumulated more rapidly in uninoculated plants than in inoculated wild-type plants (*t* test, $F_{1,6} = 7.63$; $P = 0.04$).

Growth Promotion by *S. vermifera* Is Unaffected in JA-, JA-Ile-, or TPI-Silenced Plants

Next we determined whether the growth-promoting effect of *S. vermifera* resulted from the attenuation of the growth-related costs of TPI production or from the jasmonate signals that elicit TPI. We compared stalk length of inoculated and uninoculated wild-type plants and plants transformed with antisense (as) and inverted repeat (ir) constructs of lipoxygenase 3, Thr deaminase, and TPI to silence JA, JA-Ile, and TPI levels, respectively. *S. vermifera* inoculation significantly increased stalk lengths of wild-type plants (ANOVA with repeated measures, $F_{1,23} = 76.85$; $P < 0.01$). At the

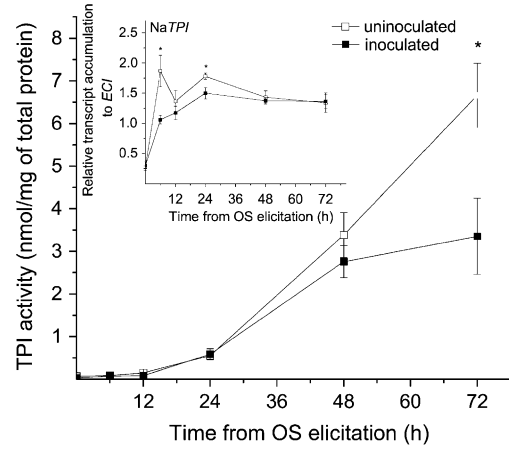

Figure 1. Association with *S. vermifera* reduces *M. sexta* OS-induced TPIs. Mean ± SE TPI activity in leaves of wild-type *N. attenuata* plants of uninduced plants (0 h) and at different time points after elicitation by wounding and applying *M. sexta* OS. White and black symbols indicate uninoculated and *S. vermifera*-inoculated plants, respectively. Inset: Mean ± SE of the relative transcript levels of Na*TPI* of the same plants. Asterisk indicates significant differences (*t* test, $P < 0.05$) between inoculated and uninoculated plants at the respective times.

end of the growth phase, 56 d after germination, inoculated wild-type plants were 6.7% taller (*t* test, $F_{1,28} = 10.03$; $P < 0.01$) than uninoculated plants (Fig. 2A). In addition, inoculated wild-type plants started to flower 1 d earlier than uninoculated plants, a difference that was highly significant (Fig. 2A; *t* test, $F_{1,27} = 11.79$; $P < 0.01$).

Similarly, *S. vermifera* significantly increased the stalk lengths of as-*lox3* and as-*td* transformed plants (Fig. 2, B and C; ANOVA repeated measures; as-*lox3*: $F_{1,23} = 107.60$, $P < 0.01$; as-*td*: $F_{1,19} = 63.22$, $P < 0.01$). *S. vermifera*-inoculated as-*lox3* and as-*td* plants started to flower 1 to 2 d earlier (Fig. 2, B and C; *t* test as-*lox3*: $F_{1,28} = 21.14$; $P < 0.01$; as-*td*: $F_{1,28} = 12.39$; $P < 0.01$), and at the end of the growth phase, *S. vermifera*-inoculated as-*lox3* and as-*td* plants were 6.7% and 3.1% taller than uninoculated plants, respectively (Fig. 2, B and C; *t* test, as-*lox*3: $F_{1,28} = 21.14$; $P < 0.01$; as-*td*: $F_{1,25} = 4.79$; $P = 0.03$). These results demonstrate that the growth-promoting effects are independent of the jasmonate signaling required to elicit herbivore defenses in *N. attenuata*.

Similar results were found in trials with ir-*tpi* plants. Inoculation significantly increased the growth of the inoculated transformed plants (Fig. 2D; ANOVA with repeated measures, $F_{1,23} = 84.04$; $P < 0.01$) so that the final stalk lengths of *S. vermifera*-inoculated ir-*tpi* plants were 6.5% taller than those of uninoculated plants (*t* test, $F_{1,28} = 13.40$; $P < 0.01$). The day flowering began did not differ between the two inoculation treatments (Fig. 2D; *t* test, $F_{1,28} = 0.18$; $P = 0.67$). We conclude that the growth-promoting effects of *S. vermifera* cannot be attributed to an alleviation of the fitness costs of TPI production.

S. vermifera Inoculation Does Not Affect the OS-Elicited Accumulation of JA and JA-Ile

Applying OS to wounded leaves elicits a dramatic JA burst that occurs in concert with a JA-Ile burst (Kang et al., 2006). These two factors have been shown to be responsible for most of the TPI transcript accumulation, as well as for the OS-induced increase in TPI activity (Halitschke and Baldwin, 2003; Kang et al., 2006). To verify the conclusions obtained from our observations of plant growth in as-*lox3* and as-*td*, we asked whether the JA and JA-Ile bursts were influenced by *S. vermifera* inoculation. No quantitative or qualitative differences were observed between the amounts of OS-elicited JA (ANOVA with repeated measures, $F_{1,7} = 1.84$; $P = 0.21$) and JA-Ile (*t* test, $F_{1,6} = 0.29$, $P = 0.61$) accumulated in the two inoculation treatments (Fig. 3).

S. vermifera Inoculation Interferes with Ethylene Signaling Independently of 1-Aminocyclopropane-1-Carboxylic Acid Deaminase Activity

The triple response assay is a rapid means of estimating the sensitivity of plants to ethylene and has been successfully used to identify ethylene-insensitive mutants (Ecker, 1995). When dark-grown seedlings are exposed to ethylene, they display shortened root and hypocotyl growth and a thickening of the hypocotyls, and the curvature of the apical hook becomes exaggerated. Since 1-aminocyclopropane-1-carboxylic acid (ACC) synthase is frequently the rate-limiting step in ethylene biosynthesis, the germination media is often supplemented with ACC to accentuate the triple response phenotype. In the triple response assay of

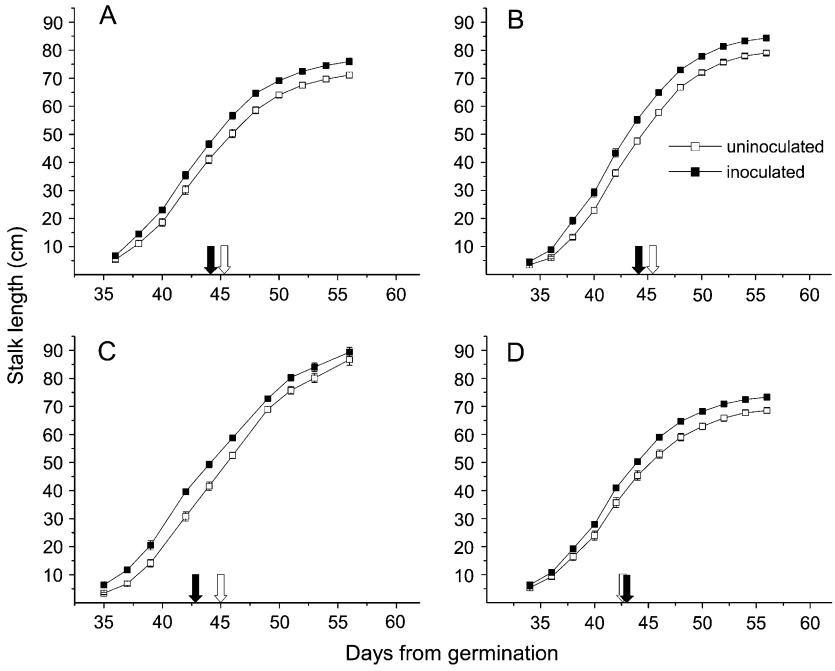

Figure 2. *S. vermifera* promotes growth of *N. attenuata*. Mean ± SE cm stalk length of uninoculated (white symbols) and *S. vermifera* inoculated (black symbols) *N. attenuata* plants at the indicated days after sowing. White and black arrows indicate the first day of flowering of uninoculated and fungus-inoculated plants, respectively. We measured wild-type (A) *N. attenuata* plants as well as transgenic plants expressing *NaLOX3* (B) or *NaTD* (C) in an as orientation and expressing *NaTPI* (D) as ir, all of which are impaired in either their JA, JA-Ile, or TPI accumulation, respectively. Repeated measures ANOVA revealed significant differences ($P < 0.01$) for all comparisons between inoculated and uninoculated plants within one genotype.

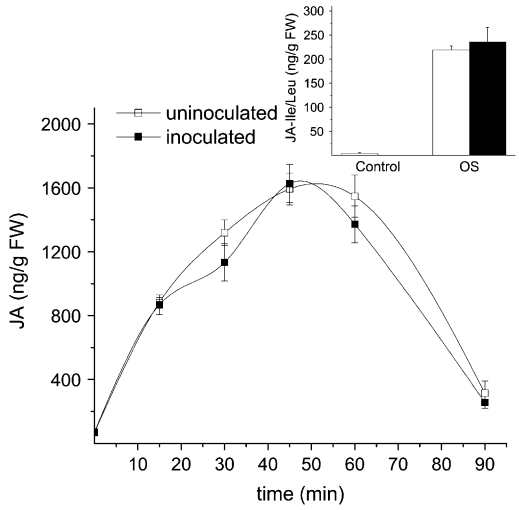

Figure 3. OS-induced accumulation of oxylipin derivatives in *S. vermifera*-inoculated and uninoculated *N. attenuata* plants. Mean ± SE JA concentrations in leaves of uninoculated (white symbols) and *S. vermifera* inoculated (black symbols) wild-type plants. Fully mature leaves (at nodal position +1) of rosette-stage plants were OS elicited and harvested from 15 to 90 min after inoculation. Untreated leaves were harvested at time 0 min. Inset: Mean + SE, JA Ile + JA Leu (JA-Ile/Leu) concentrations in leaves of uninoculated (white bars) and *S. vermifera*-inoculated (black bars) wild-type *N. attenuata*. OS-induced samples were harvested 35 min after wounding and OS application to the leaves; control samples were taken from leaves at the same nodal position of nonelicited plants.

wild-type seedlings, root and hypocotyl growth were significantly inhibited by the presence of 5 μM ACC in the media (Fig. 4; ANOVA Student-Newman-Keuls multiple comparison test, $P < 0.05$). However, inoculating wild-type seeds with *S. vermifera* prior to the triple response assay significantly reduced the inhibitory effect of ACC on root and hypocotyl length (Fig. 4; ANOVA Student-Newman-Keuls multiple comparison test, $P < 0.05$).

To determine whether the above effects are related to the ability of the fungus to degrade ACC by secreting ACC deaminase, the activity of the enzyme was assayed by measuring the amount of α-ketobutyrate produced during ACC cleavage (Penrose and Glick, 2003). By comparing the absorbance of the α-ketobutyrate standard curve to the samples we found no evidence for ACC deaminase activity in cultures of *S. vermifera*, suggesting that the reduced inhibitory effect is not related to the fungus' use of ACC as a nitrogen source.

S. vermifera Inoculation Increases Plant Performance by Inhibiting Ethylene Production

The altered growth performance of *S. vermifera*-inoculated seedlings observed in the triple response assay may be due to changes either in ethylene biosynthesis or in its perception. To examine how inoculation affects ethylene biosynthesis, we first compared the performance of inoculated and uninoculated *N. attenuata* plants transformed to silence ACC oxidase (ACO) expression in ir constructs (ir-*aco*). *S. vermifera* did not increase the performance of inoculated ir-*aco* plants as it did with wild-type plants. Stalk lengths of ir-*aco* plants were also not influenced by inoculation with *S. vermifera* (Fig. 5A; ANOVA with repeated measures $P > 0.05$). However, at the end of the growth phase, uninoculated ir-*aco* plants were 31.8% taller than uninoculated wild-type plants (compare Figs. 2A and 5A; *t* test, $F_{1,27} = 161.85$; $P < 0.01$). We therefore hypothesized that *S. vermifera*'s ability to reduce ethylene synthesis in inoculated wild-type, as-*lox3*, as-*td*, and ir-*tpi* plants was the reason for the increased growth performance of inoculated plants.

In the triple response assay, ACC significantly inhibited the root and hypocotyl growth of ir-*aco* seedlings by 79.5% and 75.7%, respectively (Fig. 5B; root: *t* test, $F_{1,6} = 21.04$; $P < 0.01$; hypocotyl: *t* test, $F_{1,6} = 3.43$; $P < 0.01$). This demonstrates that ir-*aco* plants still harbor sufficient ACO activity to induce a triple response. However, the inhibitory effect of ACC on both roots and hypocotyls was significantly reduced by preinoculation with *S. vermifera* (Fig. 5B; multiple comparisons with Student-Newman-Keuls test, $P \leq 0.05$). These results are consistent with the hypothesis that the fungus inhibits ethylene production in plants. To test this hypothesis, we compared the amount of ethylene produced in response to OS elicitation in inoculated and uninoculated plants. Measurements of ethylene emission from OS-elicited leaves revealed that uninoculated plants emitted 1.4 times more ethylene than did *S. vermifera*-inoculated plants (Fig. 6, inset; *t* test, $F_{1,14} = 8.91$; $P < 0.01$).

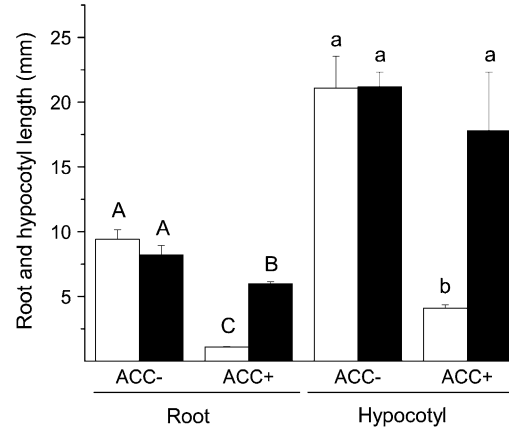

Figure 4. Triple response of uninoculated and *S. vermifera*-inoculated *N. attenuata* seedlings. Mean + SE hypocotyl and root length in mm of 10-d-old uninoculated (white bars) and inoculated (black bars) wild-type seedlings in a triple response assay. Inoculated and uninoculated wild-type seeds were germinated on media with and without the addition of 5 μM ACC. Different capital letters and lowercase letters indicate significant differences among roots and hypocotyls, respectively (ANOVA Student-Newman-Keuls multiple comparison test, $P < 0.05$).

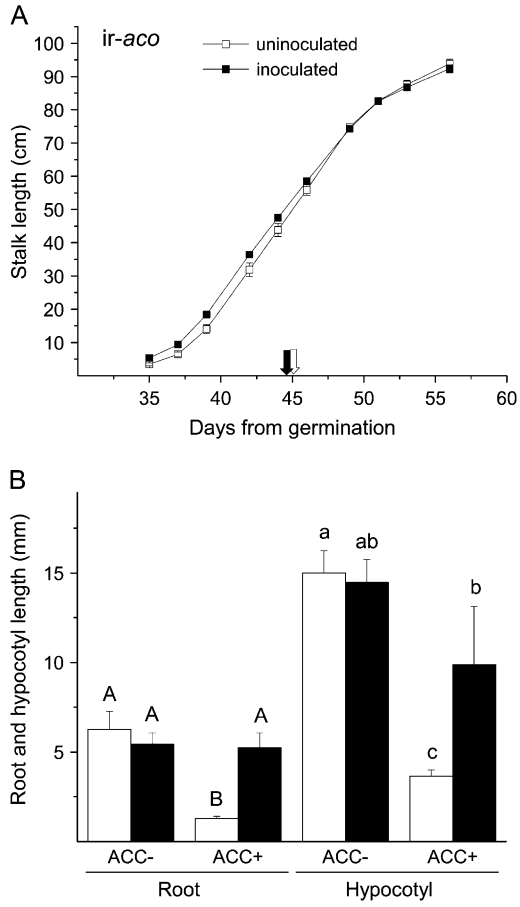

Figure 5. Growth promotion by *S. vermifera* of *N. attenuata* plants is ethylene dependent. A, Mean ± SE stalk length of uninoculated (white symbols) and *S. vermifera*-inoculated (black symbols) transformed *N. attenuata* plants expressing an *ACO* consensus region as an ir (ir-*aco*) on the indicated days after sowing. White and black arrows indicate the first day of flowering of uninoculated and fungus-inoculated plants, respectively. For comparison see stalk elongation of wild-type plants presented in Figure 2A. B, Mean + SE hypocotyl and root length of 10-d-old uninoculated (white bars) and *S. vermifera*-inoculated (black bars) ir-*aco* seedlings in a triple response assay. Inoculated and uninoculated seeds were germinated on media with and without the addition of 5 μM ACC. Different capital letters and lowercase letters indicate significant differences among roots and hypocotyls, respectively (ANOVA Student-Newman-Keuls multiple comparison test, $P < 0.05$).

To learn how the fungus inhibits ethylene production, we measured the transcript accumulation of *N. attenuata*'s ethylene biosynthetic genes by quantitative reverse transcription-PCR. OS elicitation in both uninoculated and inoculated plants resulted in the rapid accumulation of *NaACS3a* (ACC synthase) transcripts, the first committed step of ethylene biosynthesis. Maximum transcript levels attained were not influenced by inoculation (*t* test, $F_{1,8} = 0.01$; $P = 0.90$). Six hours after OS elicitation, *NaACS3a* levels began to decrease; final transcript levels of inoculated plants were significantly lower than those of uninoculated plants (Fig. 6; *t* test, $F_{1,6} = 7.39$; $P = 0.03$). In addition, we measured the transcript levels of three ACO genes, which are involved in the second committed step of ethylene biosynthesis. Compared to transcript levels in uninoculated plants, those in inoculated plants of *NaACO1* and *NaACO3* were significantly reduced following OS elicitation: by 1.9-fold after 2.5 h and 3.0-fold after 6 h, respectively (Fig. 6; *NaACO1* at 2.5 h: *t* test, $F_{1,7} = 16.72$; $P < 0.01$; *NaACO3* at 6 h: *t* test, $F_{1,8} = 8.34$; $P = 0.02$). No differences between the two inoculation treatments were measured in *NaACO2* transcripts (Fig. 6). Unlike levels of ethylene biosynthetic genes, levels of the ethylene receptor gene *NaETR1* were not affected by either OS elicitation or fungal inoculation (Supplemental Fig. S1).

Inoculation Accelerates Seed Germination

Whereas the germination of wild-type seeds on *S. vermifera*-inoculated media was significantly higher (85%; *t* test, $F_{1,5} = 9.54$; $P = 0.03$) than on uninoculated media (53%; Fig. 7), the germination rate (70%) of ir-*aco* seeds was not influenced by the presence of the fungi (Fig. 7). The presence of the ethylene scrubber, $KMnO_4$, increased the germination rates of uninoculated wild-type seeds to the level found in *S. vermifera*-inoculated seeds (*t* test, $F_{1,6} = 5.85$; $P = 0.05$). $KMnO_4$ had no effect on the germination of inoculated and uninoculated ir-*aco* seeds (Fig. 7). These results are consistent with the hypothesis that reducing ethylene in the headspace of germinating seeds accelerates germination and that inoculating plants with *S. vermifera* reduces the amount of ethylene seeds produced during germination.

DISCUSSION

S. vermifera (Sebacinales) increases the performance of *N. attenuata* plants by down-regulating ethylene production. Previously we showed that the association of *S. vermifera* increased the performance of inoculated *N. attenuata*. This growth benefit was accompanied by a decreased resistance to attack from *M. sexta* larvae, which could be attributed to the down-regulation of TPIs (Barazani et al., 2005). Here we show that the association with *S. vermifera* also reduces the transcript levels of *NaTPI* (Fig. 1, inset). Since the production of defense compounds provides a fitness benefit when plants are exposed to herbivores, but exacts fitness costs from the plant under normal growth conditions (Zavala et al., 2004a, 2004b), growth promotion by *S. vermifera* could have resulted from reducing the costs of TPI production. However, inoculating transformed lines of *N. attenuata* impaired in their expression of *NaTPI* (ir-*tpi*) increased their growth performance just as it did in wild-type plants (Fig. 2, A and D). This indicates that the beneficial effects of *S. vermifera* are not solely the result of down-regulating TPIs. Furthermore, ir-*tpi* plants flowered earlier than wild-type plants, and the flowering time of ir-*tpi* plants did not differ between the two inoculation treatments (Fig. 2D). Fitness benefits have been associated with the

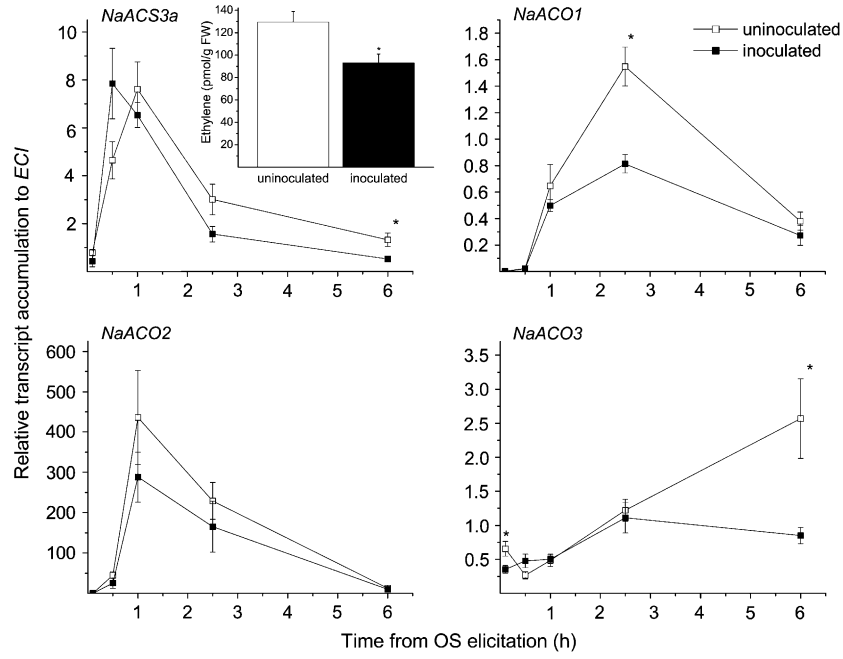

Figure 6. OS-induced ethylene emission and transcripts of ethylene biosynthesis genes in leaves of *S. vermifera*-inoculated and uninoculated plants. Mean ± SE of the relative transcript levels of *N. attenuata*'s ethylene synthesis genes *NaACS3a*, *NaACO1*, *NaACO2*, and *NaACO3* in uninoculated (white symbols) and *S. vermifera*-inoculated (black symbols) wild-type plants, at the indicated time points following OS elicitation. Asterisks indicate significant differences between fungus-inoculated and uninoculated plants (*t* test, $P < 0.05$). Inset: Mean + SE ethylene emitted by excised rosette leaves from uninoculated (white bars) and *S. vermifera*-inoculated (black bars) wild-type *N. attenuata* plants. Ethylene was accumulated in the headspace for 3 h after leaves were OS elicited. Asterisks indicate significant difference (*t* test, $P < 0.01$).

silencing of TPIs under constitutive conditions (Zavala et al., 2004a); these beneficial effects might be stronger than any effect of *S. vermifera* inoculation. Therefore, we cannot exclude the possibility that earlier flowering time of *S. vermifera*-inoculated plants might be partially caused by down-regulating TPIs.

In addition to their effect on the nutritional status of a plant, its primary metabolism, and the plants' tolerance to stress, beneficial microorganisms can increase plant growth by modifying endogenous phytohormone levels in the plant (Smith and Read, 1997; Arkhipova et al., 2005; Ryu et al., 2005; Wang et al., 2005; Madhaiyan et al., 2006). Moreover, JA and its conjugated form, JA-Ile, were shown to be involved in the establishment of AM fungi (Hause et al., 2002; Isayenkov et al., 2005). To understand whether the increase in plant performance caused by *S. vermifera* is related to changes in phytohormone signaling, we measured the performance of *S. vermifera*-inoculated transgenic lines that had been independently silenced in two steps of the oxylipin pathways. Silencing the expression of lipoxygenase 3 (Na*LOX3*; as-*lox3*) and Thr deaminase (Na*TD*; as-*td*) lowers TPI expression and increases plants' vulnerability to herbivores (Halitschke and Baldwin, 2003; Kang et al., 2006). The plant growth-promoting effects of *S. vermifera* were as evident in these jasmonate-impaired transgenic lines as they are in wild-type lines (Fig. 2), demonstrating that the growth-promoting effects and down-regulation of TPIs in inoculated plants (Fig. 1) are not mediated by alterations in JA signaling by *S. vermifera* inoculation. Further support for this hypothesis was found in measurements of the JA and JA-Ile concentrations, which did not differ between the two inoculation treatments (Fig. 3). Similarly, *P. indica* (Sebacinales), which is closely related to *S. vermifera*, had no effect on the regulation of JA- and SA-related genes in barley (Waller et al., 2005).

O'Donnell et al. (1996) have shown that the expression of proteinase inhibitor genes in tomato (*Solanum lycopersicum*) is regulated both by JA and ethylene. In *N. attenuata*, ethylene emissions increased after feeding by *M. sexta* larvae or after treatments of wounded leaves with its OS (Kahl et al., 2000). Although OS-induced nicotine levels are attenuated by ethylene (Kahl et al., 2000), a second well-described induced defense, the activity of TPI, is severely reduced in transgenic

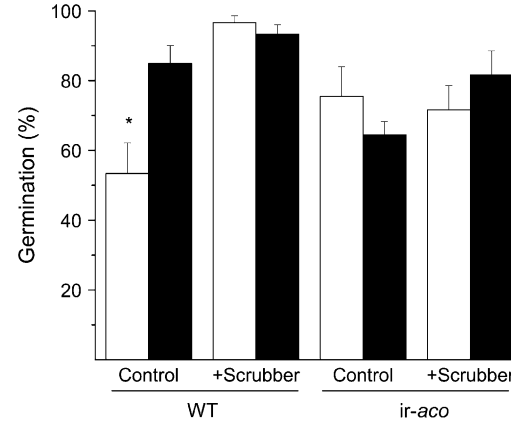

Figure 7. *S. vermifera* induces germination of *N. attenuata* by inhibiting ethylene production. Mean + SE germination rate of wild type and ir-*aco* *N. attenuata* seeds on *S. vermifera*-inoculated (black bars) and uninoculated (white bars) plates with and without the ethylene scrubber $KMnO_4$. The number of germinated seeds was determined 7 d after sowing. Asterisk indicates significant difference in germination between inoculated and uninoculated seeds within one genotype of each treatment (*t* test, $P < 0.05$).

plants impaired in ethylene synthesis (ir-*aco*), constitutively and following OS elicitation (C.C. von Dahl and I.T. Baldwin, unpublished data). Strikingly, growth promotion mediated by *S. vermifera* was lacking in fungus-inoculated ir-*aco* plants (Fig. 5A). These results are similar to those of Ryu et al. (2005), who demonstrated with several mutant lines of Arabidopsis (*Arabidopsis thaliana*) in an in vivo experimental system that growth promotion by several beneficial bacteria required ethylene signaling. The effect of silencing of defense-related genes on plant growth and fitness of *N. attenuata* has been previously discussed (Zavala and Baldwin, 2006). Here we show that inoculated as-*lox3* and as-*td* plants flowered earlier than uninoculated plants, which was not the case in ir-*aco* plants (Figs. 2 and 5A). The fact that TPI is constitutively down-regulated in all the three transgenic lines is consistent with the hypothesis that ethylene signaling, rather than TPI production, mediates the growth promotion of *S. vermifera*-inoculated *N. attenuata* plants.

We further hypothesized that plants decrease their ethylene production when inoculated with *S. vermifera*. When ACC was added to germinating seedlings, the fungus inhibited the triple response of inoculated seedlings of both wild-type and ir-*aco* plants (Figs. 4 and 5B). In addition, oxidizing ethylene with a $KMnO_4$ ethylene scrubber mimicked the effect of *S. vermifera* on the germination of wild-type seeds (Fig. 7), providing further evidence that the fungus promotes growth by manipulating ethylene production. Several beneficial microorganisms modify ethylene production by metabolizing ACC and synthesizing and secreting ACC deaminase; the cleaved ACC can then be utilized as a nitrogen source by the fungus, and by reducing ethylene production in host plants, growth is promoted (Penrose and Glick, 2003; Madhaiyan et al., 2006). Because we did not find any evidence for ACC's deaminase activity in cultures of *S. vermifera*, we measured fungus-induced changes in the plant's ethylene biosynthesis. Since OS elicitation dramatically stimulates ethylene production in *N. attenuata* (Kahl et al., 2000), we treated wounded leaves with OS and found in *S. vermifera*-inoculated plants a significant reduction in ethylene emission (Fig. 6, inset), as well as lowered transcript levels of the ethylene synthesis genes *NaACS3*, *NaACO1*, and *NaACO3* (Fig. 6), demonstrating a systemic down-regulation of ethylene biosynthesis in *S. vermifera*-inoculated plants. In a recent study by Waller et al. (2005), the ability of *P. indica* to increase plant tolerance to pathogenic attack and salt stress was associated with the increased concentration of ascorbate and the low concentration of dehydroascorbate in inoculated barley roots. Since ACO converts ACC and ascorbate to ethylene and dehydroascorbate, it is possible that down-regulating ACO genes in the inoculated roots increases the accumulation of ascorbic acid, consequently enhancing plants' tolerance to biotic and abiotic stresses.

Several other reports have demonstrated the beneficial effects of *P. indica* on different plant species (Sahay and Varma, 1999; Peskan-Berghofer et al., 2004), but it was not clear how the fungus increases plant growth and fitness. We have shown that in the *N. attenuata*-*S. verimfera* interaction, inhibiting ethylene synthesis increases plants' susceptibility to herbivorous insects while promoting plant growth. Ethylene accumulates in plants in response to different biotic and abiotic stresses. In addition, ethylene production, an early response of pathogen attack, appears to help regulate defense responses (Knoester et al., 1998; Iniguez et al., 2005; Shan and Goodwin, 2006), including inhibiting mycelia growth (Chague et al., 2006). The JA/ethylene regulated proteinase inhibitors (O'Donnell et al., 1996) are a crucial defense response to both herbivores and pathogens (Mosolov et al., 1976; Ryan, 1989; Nakagami et al., 2005). Considering TPI's dual effects, the initial steps in the interaction of *N. attenuata* with *S. vermifera* may involve a suppression of the microbial-induced ethylene-regulated defense mechanisms. Additionally, a recent study has shown that in *P. indica*-inoculated barley root, the programmed cell death hypersensitive reactions that are associated with the attack of biotrophic pathogens are repressed (Deshmukh et al., 2006), suggesting that upon colonization of plant roots with endophytic Sebacinales fungi, both ethylene and SA defense responses are suppressed. Whether this fungal-plant association is a true mutualistic interaction remains an open question that will be best addressed by experiments in *N. attenuata*'s natural habitat.

MATERIALS AND METHODS

Plant Performance

Seeds of an inbred line of *Nicotiana attenuata* Torr. ex. Wats. (synonymous with *Nicotiana torreyana* Nelson and Macbr.; wild type) as well as of several genetically transformed as and ir lines, as-*lox3* A-300-1 (Halitschke and Baldwin, 2003), as-*td* A-303-3 (Kang et al., 2006), ir-*tpi* A04-186-1 (Steppuhn and Baldwin, 2007), and ir-*aco* A03-321-10 (von Dahl et al., 2007) were germinated on Gamborg's B5 medium (Krügel et al., 2002). Methyl jasmonate-induced levels of *NaTPI* transcripts in ir-*tpi* plants are below 1% of the transcript levels observed in elicited wild-type plants. Furthermore, no TPI activity is detectable in ir-*tpi* plants regardless of the induction (Steppuhn and Baldwin, 2007).

Petri dishes were either preinoculated with *Sebacina vermifera* or left sterile. An axenic culture of *S. vermifera* (received from P. Franken, Max Planck Institute for Terrestrial Microbiology) was used to inoculate GB5 plates by preincubation in the dark at 26°C for 8 d (Barazani et al., 2005). During germination, plates were maintained at 26°C with an 11/13 h day/night cycle. Ten-day-old seedlings were transferred to Teku pots and 10 d later transferred to 1 L pots filled with B410 pot-soil mixture consisting of 95% turf and 5% clay, including 70 mg L^{-1} nitrogen, 35 mg L^{-1} phosphorus, and 75 mg L^{-1} potassium with a pH between 5.5 and 6 (Stender). Each of the genotype comparisons of uninoculated and *S. vermifera*-inoculated plants consisted of 10 to 15 pots with a single plant in each pot. About 1 month after germination, when plants had reached the elongation stage, stalk length was measured every second day and the start of flowering was recorded for each plant. About 60 d after germination, when plants stopped elongating, final stalk length was measured.

OS Elicitation Treatment

Creating standardized puncture wounds and immediately applying *Manduca sexta* larvae OS to the puncture wounds precisely mimics the transcriptional (Roda et al., 2004), proteomic (Giri et al., 2006), and metabolic (Halitschke

et al., 2001) responses of *N. attenuata* to *M. sexta* attack. Moreover, with this method, the timing of the elicitation can be standardized precisely. To elicit TPI activity, transcript accumulation, and ethylene emission, puncture wounds on the leaf blade were created with a pattern wheel on each side of the midrib and diluted OS was immediately applied to the wounds. OS were collected from *M. sexta* larvae reared on *N. attenuata* leaf diet, diluted 1:5 (v/v) with water prior to each experiment.

TPI Activity Assay

To determine TPI activity, leaf samples were harvested 3 d after OS elicitation, frozen in liquid nitrogen, and stored at −80°C until further processing. Samples were analyzed for TPI activity in an agar diffusion assay as described in van Dam et al. (2001). Levels of TPI are expressed in nanomole of inhibited trypsin proteinase molecules per milligram of total soluble protein, calculated by the clear zone of inhibitor-proteinse complex of the tested samples in reference to a standard soybean (*Glycine max*) proteinase inhibitor curve (Jongsma et al., 1994). Protein concentration was determined according to Bradford (Bradford, 1976).

Phytohormone Measurements

Leaf samples for hormone analysis were harvested at the indicated time points following OS elicitation. Approximately 300 mg of harvested leaf tissue were homogenized in 1 mL ethyl acetate spiked with 200 ng mL^{-1} [$^{13}C_2$] JA and *para* chlorogenic acid, as internal standards for JA and JA-Ile, respectively. After centrifugation at 13,000 rpm for 20 min at 4°C, extraction was repeated with 1 mL ethyl acetate. The supernatants were combined and evaporated until dryness. The dried residue was redissolved in 500 μL 70% (v/v) methanol. Prior to analysis the samples were centrifuged for 10 min at 13,000 rpm and 15 μL of the supernatant was analyzed using a Varian 1200 L triple quadrupole mass spectrometer.

For the HPLC, a Pursuit C8 column (150 mm × 2.0 mm, 3 μm particle size) was used and a gradient of water and methanol, both including 0.05% (v/v) formic acid, was the mobile phase with a flow rate of 0.2 mL min^{-1}. The mass spectrometer was operated in negative electrospray ionization mode with an argon pressure of 0.279972 Pa (=2.1 mTorr) in the collision cell. A capillary voltage of −3,200 V, a shield voltage of 600 V, and a detector voltage of 1,800 V was used. The pressure of the drying gas (N_2) was 131,005 Pa (=19 psi) at 300°C and that of the nebulizing gas (air) was 379,225 Pa (=55 psi). The most abundant and characteristic fragment ion was chosen for quantification.

Ethylene emission was measured continuously and noninvasively in real time with a laser photoacoustic spectrometer. The light source consisted of a line-tunable infrared laser and the detection device was a resonant photoacoustic cell (INVIVO). For a detailed description, see von Dahl et al. (2007). Stop-flow measurements were performed with a 250 mL cuvette that was flushed with 130 to 150 mL min^{-1} catalyzed air after the headspace of two fully mature, detached, and OS-elicited leaves (+1 and +2 nodal positions) had accumulated in the cuvette for 3 h (n = 8).

Seedling Performance Assays

We used the triple response assay to measure the effect of ACC supplementation and hence, ethylene, on the growth of uninoculated and *S. vermifera*-inoculated wild-type and ir-*aco* seedlings. Square (12 cm^2) petri dishes were filled with 80 mL of GB5, with or without 5 μM ACC (Fluka, Sigma), and the solidified agar was portioned out into two plates. Seeds (sterile or preinoculated with *S. vermifera*) were placed on the agar to germinate. The plates were stored vertically in an incubator (26°C with an 11:13 h day/night cycle); after 3 d, when the radicles emerged, the light was turned off and seedlings were grown in the dark. Each inoculation and ACC treatment consisted of four plates each with 15 seedlings. After 10 d, the lengths of roots and hypocotyls were measured.

An ethylene scrubber ($KMnO_4$) was used to test the role of ethylene in *S. vermifera*-mediated effects (Jayaraman and Raju, 1992). Seeds of wild-type and ir-*aco* plants were germinated on *S. vermifera* preinoculated or sterile GB5 media in round petri dishes (r = 4.5 cm) as described above. The open plates containing the seeds were placed in the center of a larger petri dishes (r = 7 cm). The space of the larger petri dish surrounding the smaller petri dish was filled with 50 g $KMnO_4$ beads (Profresh, Bioconservacion). Plates were maintained at 26°C with an 11/13 h day/night cycle. Germination was assessed every 24 h until all seeds were fully developed. Each treatment consisted of four replicate plates with 15 seeds per plate.

ACC Deaminase Analysis

Measurement of ACC deaminase activity was performed following Penrose and Glick (2003). For fungus culture, mycelia of *S. vermifera* were inoculated in 25 mL Luria-Bertani medium. Cultures were grown in the dark at 200 rpm, at 26°C. After 8 d, fungus mycelia were transferred to minimal medium with ACC as the only nitrogen source. Measurements of enzyme activity were conducted on two separate cultures as described by Penrose and Glick (2003).

RNA Isolation and mRNA Expression

Fully mature leaves (at nodal position +1) of rosette-stage plants were elicited with OS as described above. Leaves were collected at different time points after the elicitation (for ethylene biosynthesis genes: 0 nonelicited, 30, 60, 150, and 360 min; for *NaTPI*: 0, 6, 12, 24, 48, and 72 h), immediately frozen in liquid nitrogen, and kept at −80°C until further processing. For each time point, one leaf was harvested from five different elicited plants. Total RNA was extracted using TRI reagent (Sigma). cDNA was synthesized from 20 ng of total RNA as described by Schmidt et al. (2005) using the Taqman reverse transcription reagent kit (Applied Biosystems). Analysis of the relative expression of ethylene biosynthesis and perception genes was performed using primer pairs and fluorescent dye-labeled probes for *NaACS3a* (AY426752), *NaACO1* (AY426756), *NaACO2* (EF123109), *NaACO3* (EF123111), and *NaETR1* (EF203416), as described by von Dahl et al. (2007). Analysis of *NaTPI* (AF542547) was performed using primers and fluorescent dye-labeled probes as described by Zavala et al. (2004a). For each analysis, a linear standard curve, threshold cycle number versus Log (designated transcript level), was constructed using a series dilutions of a specific cDNA standard; the levels of the transcript in all unknown samples were determined according to the standard curve. A *N. attenuata sulfite reductase* (*ECI*), which is a housekeeping gene involved in plant sulfur metabolism and has been shown to have constant levels of transcript by both northern blotting and quantitative PCR, after wounding and OS elicitation (Wu et al., 2007), was used as an internal standard for normalizing cDNA concentration variations. Real-time PCR was performed on a SDS7700 (Applied Biosystems) using the quantitative PCR reagent kit (Eurogentec); for a detailed description see Schmidt et al. (2005).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Transcript accumulation of ethylene receptor gene *NaETR1* in the leaves of OS-elicited uninoculated and *S. vermifera*-inoculated *N. attenuata*.

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Manuscript V

Volatile signaling in plant-plant interactions: “talking trees” in the genomic era

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Caroline C. von Dahl, and Cathy A. Preston

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This review summarizes the recent literature on plant-plant communication via released volatile organic compounds. Ian T. Baldwin wrote the manuscript with help from Rayko Halitschke on the section describing signal properties. Anja Paschold helped with the section on recent literature addressing plant-plant communication in response to herbivory and designed the figures. I contributed the section on ethylene signaling between competing plants and in response to herbivory, while Catherine A. Preston helped with the summary of the literature on *Artemisia tridentata* and *Nicotiana attenuata.*

PLANT VOLATILES

REVIEW

Volatile Signaling in Plant-Plant Interactions: "Talking Trees" in the Genomics Era

Ian T. Baldwin,[1]* Rayko Halitschke,[2] Anja Paschold,[1] Caroline C. von Dahl,[1] Catherine A. Preston[3]

Plants may "eavesdrop" on volatile organic compounds (VOCs) released by herbivore-attacked neighbors to activate defenses before being attacked themselves. Transcriptome and signal cascade analyses of VOC-exposed plants suggest that plants eavesdrop to prime direct and indirect defenses and to hone competitive abilities. Advances in research on VOC biosynthesis and perception have facilitated the production of plants that are genetically "deaf" to particular VOCs or "mute" in elements of their volatile vocabulary. Such plants, together with advances in VOC analytical instrumentation, will allow researchers to determine whether fluency enhances the fitness of plants in natural communities.

Plants excel at gas exchange: They can literally build forests from CO_2 taken from the air at about 120 Pg C $year^{-1}$, half of which is respired back to the atmosphere. Up to 36% of the assimilated carbon is released as complex bouquets of VOCs (*1*). Although some of these VOCs may be mere waste, others mediate various pollination and defense mutualisms with animals. These VOC-mediated interactions of plants with organisms of higher trophic levels suggest that they communicate similarly with each other (*2*). Two decades ago, researchers serendipitously discovered changes in herbivore resistance and secondary metabolites in plants ("receivers") growing adjacently to herbivore-attacked plants ("emitters"). Because in some experiments results were best explained by the aerial transfer of information (*3*), the phenomenon was popularly dubbed "talking trees." This phrase seems unfortunate, because selection most likely favors plants that "eavesdrop" on VOCs released from neighbors and respond by tailoring their phenotypes to enhance their own fitness.

What Are Plants Talking About?

An obvious conversation topic concerns impending attack from mobile herbivores, and most VOC-elicited responses have been interpreted accordingly. Measures of herbivore performance have been broadened to include the elicitation of various direct plant defenses (e.g., phenolics, alkaloids, terpenes, and defense proteins). Indirect defenses have also attracted attention, including food rewards that increase predation pressure on herbivores (*4*) and VOCs that help predators or parasitoids locate feeding herbivores (*5*, *6*). Moreover, the signal cascades that elicit direct and indirect defenses have been scrutinized (*7*, *8*) as have transcriptional responses (*9*–*12*) (Fig. 1).

VOC exposure alone, without actual herbivore attack, may directly increase the production of defenses. Alternatively, VOC exposure may allow nearby plants to ready their defenses for immediate use once the herbivores move from the neighboring plant to attack the "listening" receiver. Exposure to volatiles from damaged sagebrush primes the elicitation of defensive proteinase inhibitors (PIs) in wild tobacco, and exposed plants subsequently receive less damage (*13*–*15*) (Fig. 2). Corn seedlings previously exposed to either individual components or to the entire blend of VOCs released from herbivore-attacked seedlings responded to simulated herbivory with increased VOC production and higher jasmonate (JA) accumulations compared with the responses of unexposed plants (*8*). Whether these enhanced VOC emissions protect corn seedlings remains to be determined. The priming of defense cascades may benefit plants that would incur fitness costs by activating defense responses (*16*), particularly in the absence of herbivore attack (*17*). If VOC exposure directly elicited defense responses, receiver plants would incur similar fitness costs without being damaged. Hence, plants that avoided investing fitness-limiting resources in the production of costly defenses before an herbivore arrives, but were able to prime defense metabolism to launch defense responses when attacked, could realize a fitness benefit over plants that "ignored" the information coded in the VOCs emanating from their damaged neighbors.

The use of microarrays that monitor a large fraction of the plant's transcriptome can free analysis from observer bias about plants' conversation topics and identify selective pressures other than impending attack from mobile herbivores, which volatile signaling could be used to anticipate. Herbivores frequently transmit pathogens, and the elicited responses may concern attack by impending pathogens more than attack by herbivores (*18*). The relentless competition with other plants for resources that cannot be readily hoarded (such as light and nutrients) is likely the most important selective force for plants. Plants are able to anticipate impending competition through far red (FR) light signals and changes in the photon flux of blue light transmitted through their neighbors' canopies. These light signals are perceived by photoreceptors (e.g., phytochrome B) and elicit a complex of traits known as the shade-avoidance syndrome (SAS) (*19*). Experiments with tobacco plants transformed with a mutant ethylene receptor (*etr1-1*), which inhibits ethylene perception, have demonstrated that ethylene-insensitive tobacco could not respond rapidly to FR signals and consequently was outcompeted by wild-type plants (*20*). At concentrations apparently possible in dense plant canopies, ethylene by itself elicits the SAS (*21*). Similarly, exposure to unidentified VOCs from barley cultivars changes the allocation of biomass between roots and shoots without influencing biomass production of receiver barley genotypes (*22*), a re-allocation that may influence competitive ability. Thus, responses to the most important environmental factors in a plant's life may be anticipated by signals from neighboring plants. Almost anything can be a signal as long as it can be perceived and provides reliable information.

What Does It Take to Be a Signal?

Four steps characterize the transfer of VOC signals between plants: the release of the signal by the emitter plant and its transport, absorption, and perception by the receiver plant (Fig. 1). All are influenced by the signal's properties and its biological context. Most research on signal release has focused on the activation of biosynthetic enzymes and their substrate supply. The biochemical control mechanisms for the major VOC constituents are rapidly being clarified (Fig. 1). However, the release of foliar VOCs is also controlled by their physicochemical properties (*23*): Volatility is deter-

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mined by partitioning the compound between the "liquid" phase of the leaf and the atmosphere, whereas molecular size and stomatal aperture constrain diffusive transport from the leaf into the air surrounding the leaf, its headspace. Once released into the headspace of the emitter, the potential signal has to be transported to receivers. Direction and dynamics of this transport are dictated by temperature, convective transport, and wind for above-ground signaling or water for below-ground signaling. Small highly volatile compounds (e.g., ethylene, methanol, isoprene, acrolein, methacrolein, and some monoterpenes) diffuse rapidly into the headspace and are diluted in the atmosphere (Fig. 2). For such compounds, signaling function is likely limited to the foliage of the emitter (as a systemic within-plant signal) and of neighbors with intertwined canopies. Heavier compounds with less volatility, such as terpene alcohols, methyl jasmonate (MeJA), aromatic compounds including methyl salicylate (MeSA), and green-leaf volatiles (GLVs), are more likely to function as signals over longer distances, because their comparatively slower dispersal allows development of plumes of higher concentrations (*24*) that may be carried farther as intact parcels by turbulent flow (Fig. 2). During transport, some VOC species are oxidized or otherwise processed in the atmosphere (*1*), possibly causing dilution but also activation. The concentration gradients, which ultimately regulate the receiver's exposure, remain largely uncharacterized. An example of a characterized concentration gradient comes from a study of corn seedlings that release the volatile sesquiterpene (*E*)-β-caryophyllene into the soil from their roots, a below-ground plume used by entomopathogenic nematodes to locate root-attacking beetle larvae (*25*).

Signal volatility and diffusion rates, as well as the stomatal conductance of receiver plants, define the last steps in the signal transfer process: adsorption at the plant surface and uptake into the leaf via stomatal openings or cuticle diffusion. The low concentration gradient between atmosphere and leaf during the adsorption step amplifies the effects of the signal's physicochemical properties. Transport into the receiver leaf is influenced by stomatal conductance. The limited air volume of a sealed chamber increases VOC concentrations and also reduces CO_2 once the chambers are illuminated because of photosynthetic carbon fixation. Under such conditions, plants increase the number of open stomata, enhancing exposure of mesophyll cells to the VOCs. Therefore, sealed chambers are likely to influence the responsiveness of receiver plants, and studies that use them are more likely to report ecologically insignificant results.

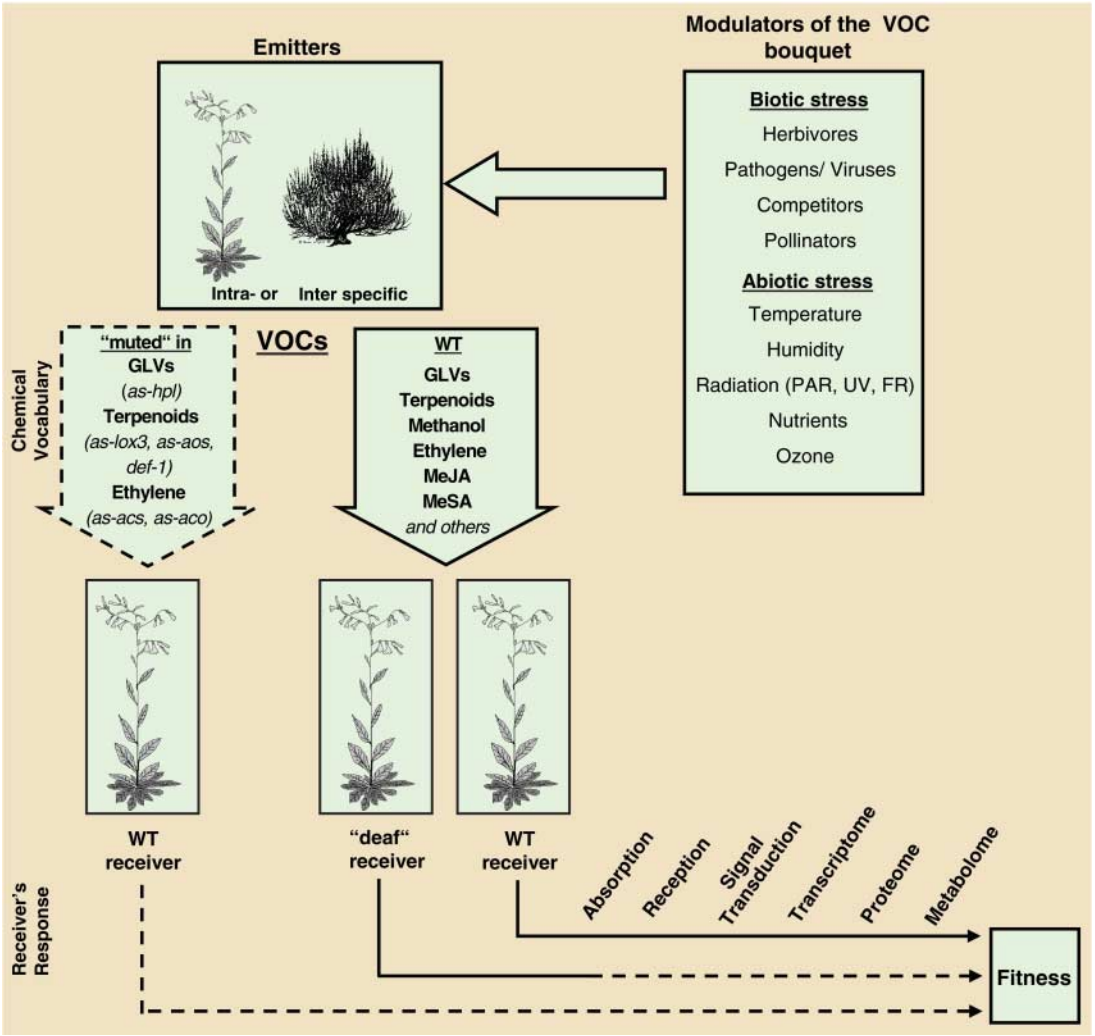

Fig. 1. Scheme of plant-plant interaction mediated by VOCs emphasizing the use of genetically manipulated plants to investigate the mechanisms underlying this process. Plants (e.g., wild tobacco) can be exposed to VOCs released from either conspecifics or from emitters of different species (e.g., sagebrush). The VOC bouquet of stressed plants consists of GLVs, terpenoids, MeJA, MeSA, methanol, ethylene, and other substances (*32*). Various biotic and abiotic stress factors modulate the chemical vocabulary emitted in quantity, quality, and timing. If the signal is recognized by the receiver plant, it may respond with changes in its signal transduction, transcriptome, proteome, and metabolome, which may or may not result in functionally significant changes in its fitness (→). Comparing responses to wild-type (WT) emitter plants with responses to mute emitters (⇢) whose VOC bouquet is deficient in one or more VOCs allows researchers to identify compounds mediating the interaction between emitters and receivers. In addition to insertional mutants [e.g., *def-1* (*33*)], various transgenic lines are generated by the expression of endogenous genes in antisense (as) orientations to silence enzymes necessary for eliciting or synthesizing VOCs, such as hydroperoxide lyase [HPL (*34*, *35*)], lipoxygenase [LOX3 (*36*)], allene oxide synthase [AOS (*35*)], 1-aminocyclopropane-1-carboxylic acid synthase [ACS (*37*)], or 1-aminocyclopropane-1-carboxylic acid oxidase [ACO (*38*)]. These lines represent possible mute emitters. Deaf receiver plants, such as the *etr1-1* line (*39*) impaired in functional VOC receptors for individual substances, could be used to verify each individual VOC's bioactivity.

Once a VOC enters the leaf, a response will only occur if the compound is "active," a poorly understood condition. Several proposed between-plant signals have hormone or hormone-like functions, including MeSA (*26*), GLVs (*8*, *9*, *27*), ethylene (*28*), and MeJA (*29*). However, proof that any of these are released and transported to receiver plants in quantities sufficient to elicit responses under natural conditions is either lacking or belies a signal function (*10*, *13*, *30*). Although most studies of bioactivity have examined whether the presence of a VOC elicits a response, removing certain components from a volatile bouquet can also elicit a response. The removal of GLVs from the wound-induced volatile blend by silencing hydroperoxide lyase strongly influenced the regulation of gene expression in neighboring conspecific tobacco plants (*10*). In other words, plants may respond to the "sounds of silence."

A class of electrophilic α,β-unsaturated carbonyl compounds represents potent regulators of gene expression (*11*). Although exposure to these highly volatile compounds increased the production of endogenous phytohormones, their activity was partially independent of the JA, SA, and ethylene signal cascades. A redox-based signal process, generated by the depletion of cellular reductants resulting from the electrophile reactivity of these compounds, suggests a mechanism for their activity that resembles the activation of the regulatory protein for pathogen defense, NPR1 (*31*). Similar processes may provide the basis of a general chemical "sense," which may have predated the evolution of receptors for particular volatiles.

Ecological Realism: "Deaf" and "Mute" Plants to the Rescue

Constitutive and herbivore-induced VOC emissions are influenced by a variety of abiotic factors [nutrient availability, temperature, wind, ultraviolet (UV) radiation and photosynthetically active radiation (PAR), and ozone exposure]. To lessen this variability, most studies of plant-plant signaling have been performed in the laboratory under experimental conditions (sealed or low air-flow chambers) that maximized the probability of detecting responses in receiver plants by increasing exposure [reviewed in (*10*)]. Although this work has shown that plants respond to being fumigated, its ecological relevance will remain unclear until the responses are verified in open-grown plants.

One solution to the problems of ecological realism in between-plant signaling studies is to use mutants or transgenic plants whose ability to either release or perceive particular components of the wild-type volatile blend is deficient. The use of "mute" emitters (*10*) allows complex herbivore-induced VOC blends to be dissected (Fig. 1). Complementation studies, in which synthetic constituents supplement the volatile blend to determine whether the receivers' response is subsequently restored, confirm function. The biosynthetic pathways contributing constituents to the herbivore-induced volatile bouquet and their regulatory cascades represent possible genetic targets. Mutants whose

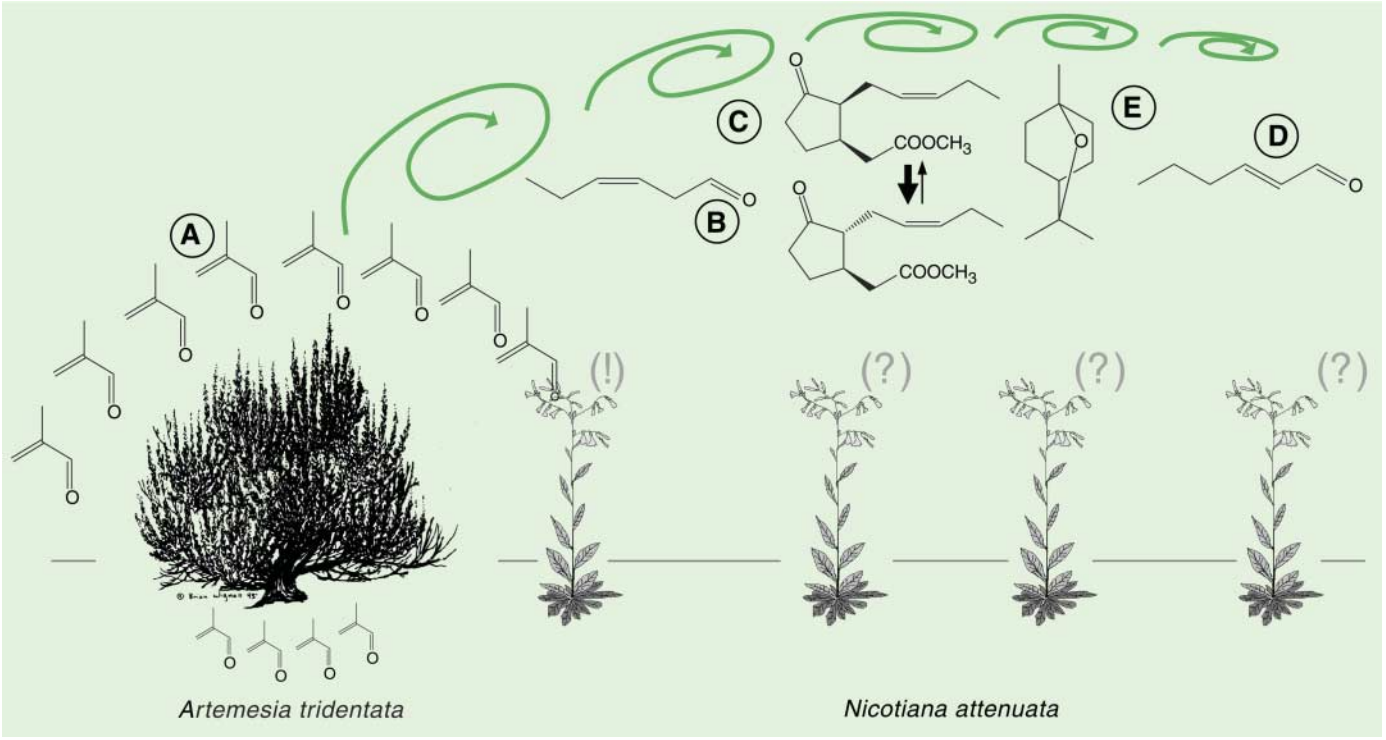

Fig. 2. Aerial interaction of the wild tobacco (*Nicotiana attenuata*) and sagebrush (*Artemisia tridentata tridentata*) (*40*) is the best-documented example of between-plant signaling via above-ground VOCs in nature (*14*, *15*, *41*). When transplanted to within 15 cm of clipped sagebrush, tobacco plants suffered less herbivory and produced more seed capsules than did plants transplanted adjacent to undamaged sagebrush. Damaged sagebrush releases a variety of VOCs, which are composed of highly volatile substances that disperse by diffusion, namely, methacrolein (A) and less volatile compounds such as GLVs [e.g., *cis*-3-hexenal (B) and *trans*-2-hexenal (D)], oxygenated monoterpenes [e.g., cineole (E), thujone, and camphor] and the epimers of MeJA (C), which are likely transported by turbulent flow in fragmented plumes. The plume from damaged sagebrush is highly enriched in the *cis* epimer of MeJA, which is thermodynamically unstable but putatively more biologically active than the *trans* epimer (*14*, *30*, *42*). Hence, MeJA was the most obvious candidate for the volatile signal mediating the response; subsequent studies were unable to confirm that either epimer of MeJA elicited known herbivore defenses when applied in quantities relevant to those released by damaged sagebrush (*30*, *42*). Rather than directly eliciting defenses, exposure to volatiles from excised sagebrush foliage (and two constituents of its aromatic headspace: *trans*-2-hexenal and methacrolein) primes defense responses, so that plants increase the production of their defense protein, PI, faster when attacked (*13*). The progress in this system highlights the difficulty of predicting how plant-plant signaling functions from first principles.

herbivore- or wound-induced vocabularies have been modified by silencing genes involved in either the biosynthesis of particular volatiles or the oxylipin signal cascade represent potential mute emitter plants (Fig. 1).

Mutants whose perception of specific VOCs is impaired ("deaf" plants) represent another tool for analyzing the consequences of VOC signaling as illustrated by the ethylene-insensitive tobacco plants, *etr1-1*. The produce industry long ago developed a sophisticated ethylene trapping and releasing technology, but the first clear demonstration of the functional significance of ethylene signaling in competitive interactions required plants that were "deaf" to this VOC (*20*). Receptors for most of the herbivore-induced VOCs remain to be discovered, but transcriptional responses to VOC exposure can be used in mutant screens to identify new VOC receptors. Identification of these genetic elements and the creation of VOC-reporter plants [with β-glucuronidase (GUS) or green fluorescent protein] will allow researchers to readily determine the quantity of signals that are perceived by receivers at different distances from an emitter. Combining deaf and mute plants with wild-type plants in natural settings will clarify the relevance of VOC signaling for a plant's performance and/or fitness in the real world. Because differences in performance among plants that are unable to produce or perceive certain volatiles are likely to be subtle, the analysis will likely require long-term studies in natural settings. The more deaf plants that are available to complement the growing list of available mute plants, the more tools researchers will have to fully evaluate the significance of volatile signaling among plants in natural settings. These experiments will determine whether being a native speaker enhances a plant's fitness in its community.

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REVIEW

Plant Volatile Compounds: Sensory Cues for Health and Nutritional Value?

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Plants produce many volatile metabolites. A small subset of these compounds is sensed by animals and humans, and the volatile profiles are defining elements of the distinct flavors of individual foods. Flavor volatiles are derived from an array of nutrients, including amino acids, fatty acids, and carotenoids. In tomato, almost all of the important flavor-related volatiles are derived from essential nutrients. The predominance of volatiles derived from essential nutrients and health-promoting compounds suggests that these volatiles provide important information about the nutritional makeup of foods. Evidence supporting a relation between volatile perception and nutrient or health value will be reviewed.

Plants are capable of synthesizing tens to hundreds of thousands of primary and secondary metabolites with diverse biological properties and functions. Plant volatile organic compounds (defined hereafter as volatiles) generated from both primary and secondary metabolites are generally low molecular weight lipophilic compounds (*1*, *2*). More than 7000 flavor volatiles have been identified and cataloged from foods and beverages (*3*, *4*). Many volatiles are produced in plant tissues at specific developmental stages—for example, during flowering, ripening, or maturation. Although a single fruit or vegetable synthesizes several hundred volatiles, only a small subset generates the "flavor fingerprint" that helps animals and humans recognize appropriate foods and avoid poor or dangerous food choices.

Although perception of flavor is often described as a combination of taste and smell (*5*), appearance, texture, temperature, mouth feel, and past experience also play major roles in flavor perception, indicating that multiple distinct sensory inputs are processed to generate the overall sensation (Fig. 1). Integration of this sensory information in the brain ultimately results in a flavor preference or aversion with a strong influence on subsequent perception and behavior. Studies of flavor preferences and aversions suggest that flavor perception may be linked to the nutritional or health value associated with the perceived foods (*6*–*11*). For example, fatty acids that stimulate taste responses are essential long-chain cis-polyunsaturated fatty acids rather than nonessential saturated fatty acids (*11*). Flavor preferences begin to develop before birth and develop rapidly in the newborn

3. Discussion

Plants emit a multitude of VOCs, such as GLVs, terpenes, ethylene, MeOH, isoprene, and acetone, with hypothesized and demonstrated functions (Gershenzon, 2007; Sharkey, 1996). An evolutionary origin of VOC emissions as by-products of metabolic pathways, which later have evolved secondary signaling functions, has been suggested (Peñuelas and Llusià, 2004; Sharkey, 1996). As much as it crucial for the plant to tell a meaningful signal from the background noise it is a challenge for scientists to identify these signals. Advances in analytical techniques including improved sampling procedures and increased detector sensitivity have led to discoveries of previously unnoticed emissions, like the recently described plant-derived emission of methane (Keppler *et al.*, 2006). While these advances clearly increase the knowledge about the release of VOCs the characterization of 'meaningful signals' does not necessarily improve at the same pace. Detailed analysis of volatile signals requires the investigation of their emissions in the context of the ecological circumstance and their manipulation by over-expression or gene-silencing techniques. MeOH and ethylene emissions and their functional relationships are the subject of the presented manuscripts.

3.1. Deciphering the role of MeOH

MeOH is a product of pectin methyl esterase (PME) activity on plant cell wall pectins. PME is active during plant development strengthening the cell wall once a leaf has reached maturity (Galbally and Kirstine, 2002). A growth-promoting effect of MeOH was identified in the early 1990s and received attention of many research laboratories. Exposing C3 plants to 20-50% aqueous MeOH significantly increased their growth and yield in arid environments (Nonomura and Benson, 1992). The increased yield achieved by MeOH exposure was of agricultural interest but the applied concentrations which extremely exceeded constitutive MeOH levels or induced MeOH emissions elicited by wounding or herbivore feeding (Nemecek-Marshall *et al.*, 1995; Peñuelas *et al.*, 2005), were too high to reveal physiologically or ecologically relevant functions in the plant. Although the mechanism underlying the process of MeOH emissions was known and at least one function -- the highly artificial growth-promoting effect -- was described, MeOH as a by-product of pectin demethylation was considered a metabolic waste-product. Interestingly, the herbivore-induced MeOH emissions are specifically regulated, triggered by characteristic elicitors (basic pH), and accompanied by protein activity and transcript regulation of PME (*Manuscript I*). This specific regulation suggests a role for PME activity during the induced defense response to herbivory in *N. attenuata*. Pectin demethylation by PME will cause changes in cell wall fluidity when demethylated pectin molecules are cross-linked by calcium bridges; this potentially changes the Ca^{2+} concentration in the apoplast (Maffei *et al.*, 2004). Furthermore, demethylated pectin can be degraded by pectinolytic enzymes (Willats *et al.*, 2001). Polygalacturonases (PG),

the most abundant pectin cleaving enzymes, have the capacity to generate oligosaccharide fragments with physiological functions, including the activation of proteinase inhibitors (PI) (Bishop *et al.*, 1981). Finally, the PME-derived MeOH emission in response to herbivory acts as a signal compound in plant-herbivore interactions. Exposure to naturally emitted MeOH concentrations decreased the abundance of active PIs in *N. attenuata* plants and thereby increased the plant's susceptibility to *M. sexta* larvae (*Manuscript I*). This suggests a putative signaling function of MeOH during herbivore attack.

Figure 2. Signaling and priming within and between plants.

Signals of diverse origin can control the responses within a plant: **a.** endogenous signals originating within the local leaf or a leaf connected by the vascular system (the latter being depicted), **b.** airborne signals perceived by systemic leaves of the same individual circumventing vascular restrictions, and **c.** external signals released by a different individual.

Priming represents a possible mechanism in plants to respond to less reliable external signals. Perception of an external signal (b or c) can enhance or fasten the response to a following internal signal (a) input. This process of increased sensitivity or responsiveness could have facilitated the evolution of plant-plant communication, whereby a non-self external signal (c) primes the response to the following perception of an internal signal (a).

The pharmacological complementation of inverted repeat (ir) plants silenced in NaPME activity (ir-*pme*) will help to identify the relevant compound, PME educt or product, that influence a plant's resistance to herbivores. ir-*pme* plants have been generated and led to significant discoveries. Silencing of PME in *N. attenuata* reduced the MeOH release in response to *M. sexta* feeding demonstrating the NaPME-mediated demethylation of pectin as the source of herbivore-induced MeOH emissions. Surprisingly, ir-*pme* plants show increased susceptibility to caterpillar feeding despite the abolished production of herbivore-induced MeOH (Evelyn Körner, Caroline C. von Dahl, and Ian T. Baldwin unpublished data). These results, although demonstrating the involvement of NaPME in *N. attenuata*'s defense response, question the proposed signaling

function of MeOH directly activating defense responses in the plant (signal type a and b, Figure 2). More likely, signaling within the plant by non-volatile PME products mediates the PME-dependent resistance (signal type a, Figure 2). The release of oligogalacturonic acids (OGAs) is facilitated by PME activity and OGAs have been shown to be transported systemically and elicit PI activity in tomato plants (Bishop *et al.*, 1981; McDougall *et al.*, 1992). Further work is necessary to dissect the role of PME-dependent defense responses by integrating internal (OGAs) and external signals (MeOH), their perception within the damaged plant (type a and b), and their perception by neighboring plants (signal type c, Figure 2).

3.2. Ethylene: Jack of all trades, master of none?

Ethylene, one of the classical phytohormones, regulates growth and development and coordinates stress responses to the abiotic and biotic environment. These processes share certain regulatory modules like phytohormones and mitogen-activated protein kinase (MAPK) activity (Zhang *et al.*, 2006) but ultimately have little in common. Flower longevity and herbivore-induced nicotine accumulation, are regulated by the same hormone -- ethylene -- and are dramatically altered in plants impaired in ethylene biosynthesis or perception (*Manuscript III*). However, flower abscission of Arabidopsis plants is accompanied by cell wall degrading processes in the abscission zone (Bleecker and Patterson, 1997) and the defense response of *N. attenuata* is marked by increased nicotine accumulation and PI activity in the local leaf (*Manuscript II*).

Plants orchestrate complex physiological processes regulated by ethylene by the specific activation of different isoforms of ACC synthase (ACS) and ACC oxidase (ACO) in response to particular developmental and stress cues (Wang *et al.*, 2002). The two committed enzymes of ethylene biosynthesis, ACS and ACO, are encoded by multigene families each consisting of at least four members in *N. attenuata* (*Manuscript III*). Herbivore-induced ethylene production in *N. attenuata* is induced by OS-derived elicitors of *M. sexta* larvae, FACs, and is regulated by the transcription of distinct ACS and ACO isoforms. OS-induced ethylene emissions are partially regulated by the transcript levels of *NaACS3a*, *NaACO2a*, and *NaACO3* resulting in a specific herbivore-induced ethylene biosynthesis that is clearly distinguished from the wound response, which in turn was accompanied by slightly increased transcript levels of *NaACO1* and *NaACO3* (*Mansucript III*). How plants and specifically *N. attenuata* regulate ethylene biosynthesis at the level of transcription and perception is thoroughly discussed in *Manuscript III*. In the following, an attempt was taken to discuss strategies to integrate a broad range of responses influenced by a single signal, once it is elicited and synthesized, without compromising its specificity in the context of ethylene and *Manuscript II* to *V*. A thorough discussion of the role of ethylene in plant-insect, plant-mycorrhizae and plant-plant interactions is given in *Manuscript II* to *V* and will not be repeated here.

3.2.1. Concentration-dependent regulation of plant growth and development

Ethylene has long been recognized as a growth inhibitor and with the use of the triple response assay, a fast and easy screen for ethylene perception, mutants that do not show stunted growth in the presence of ethylene in the dark were isolated (Guzmán and Ecker, 1990). Stems and petioles of certain plant species can retain this response pattern and show inhibited growth in response to ethylene (Smalle and van der Straeten, 1997). However, positive growth effects have been observed. While low ethylene concentrations, typically below 0.1 µl L^{-1}, stimulate vegetative growth (leaf, stem, and root elongation) in tobacco (*N. tabacum*), wheat (*Triticum aestivum*), and *Arabidopsis thaliana* (Pierik *et al.*, 2006). Flooded plants show ethylene-dependent growth responses including a more upright petiole angle and increased petiole elongation at high levels of ethylene (Voesenek *et al.*, 2006). Ethylene's low solubility in water leads to a containment of ethylene in flooded plant tissue, where it accumulates to 20-fold higher concentrations within the first hour of submergence (Voesenek *et al.*, 2006). Ethylene levels passively increase in flooded *Rumex palustris* plants, whereas enhanced biosynthetic activity causes the increase in ethylene concentration in rice (Voesenek *et al.*, 2006). Pierik *et al.* (2006) proposed a biphasic model to explain these differential responses to ethylene with low levels of ethylene-promoting and high levels of ethylene-inhibiting growth responses. The authors further hypothesize that the range of ethylene concentrations for either response depends on environmental conditions, internal signals, and species-specific characteristics, e.g. species' habitat and life history.

The germination of *N. attenuata* seedlings is inhibited in the presence of ethylene and the reduction of ethylene concentrations, by the presence of an ethylene scrubber ($KMnO_4$) or by association with *S. vermifera*, resulted in higher germination rates (*Manuscript IV*). Growth inhibition by ethylene was also observed at the whole plant level; inoculation with *S. vermifera* promotes the growth and fitness of wild type tobacco plants, but not in ir-*aco* plants impaired in ethylene signaling (*Manuscript IV*). These growth responses suggest that *N. attenuata* plants increase their vegetative growth in responses to decreased ethylene concentrations (*Manuscript IV*).

Plants in dense stands perceive changed R:FR light ratios caused by light reflections on leaf surfaces of proximate neighbors that trigger SAS (Ballaré *et al.*, 1990; Figure 1a). This growth response is promoted by ethylene that accumulates in plant gaps of dense stands. Ethylene alone can trigger enhanced shoot and petiole elongation of tobacco plants (Pierik *et al.*, 2004). The ethylene-dependent growth responses elicited in *N. attenuata* in association with *S. vermifera* and in *N. tabacum* during SAS do not only differ in the triggered growth-response due to altered ethylene concentration but differ in the spatial localization of produced and perceived ethylene. In the interaction with the endophytic fungus a reduced ethylene biosynthesis inside the plant (signal type a and b, Figure 2) leads to increased growth (*Manuscript IV*), while in the SAS

response the perception of ethylene produced by neighboring plants (signal type c, Figure 2) increased stalk and petiole elongation.

3.2.2. Localized regulation of ethylene biosynthesis

Water stress responses of flooded tomato plants include increased production of ethylene in the shoots. This ethylene biosynthesis of flooded tomato plants shows a remarkable level of localization of the individual biosynthetic steps. 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene, is produced by a ACS in the roots and transported to the shoots where it is converted to ethylene by ACO activity (Grichko and Glick, 2001). Thus, distinct activation of ACS and ACO regulates ethylene biosynthesis and localizes the ethylene-dependent responses in flooded tomato plants in the shoot. In contrast, the elicitation of herbivore-induced ethylene in *N. attenuata* shows no tissue-specific separation of ethylene biosynthetic processes. Transcript accumulation of both key enzymes is induced in the herbivore-attacked leaf tissue, where the induced ethylene is produced and released; yet no ethylene emissions in response to leaf herbivory was detected in root tissue (*Manuscript III*, Caroline C. von Dahl and Ian T. Baldwin unpublished data). Despite the fungal infection of the roots, the reduction of herbivore-induced ethylene biosynthesis in the leaves of *S. vermifera*-inoculated plants is accompanied by reduced ACS and ACO activity indicating a co-localization of ACS and ACO activity (*Manuscript IV*).

Figure 3. "Permaflower" phenotype of ethylene-insensitive *N. attenuata* plants.
The average flower lifetime of ethylene-insensitive *N. attenuata* plants was extended up to 3-fold in comparison to wild type (WT) floral longevity. Plants with the strongest ethylene-insensitive phenotype (35S-etr1b) displayed permaflowers that retained white and turgid corollas long after pollination and well into the seed-filling stage of capsule development. WT corollas start senescing 3 days after anthesis and usually abscise their corollas before capsule development. Photographer: Danny Kessler

A strong localized effect of ethylene signaling is involved in flower development. Senescence and abscission determines flower longevity and both traits are under the control of ethylene signaling (Patterson and Bleecker, 2004). Longevity enhances the floral display and plays an important role in reproductive ecology of plants by increasing the number of potential pollinator visits and pollination success. At the same time extended floral displays may incur fitness costs by prolonging resource allocation to the reproductive tissue and increasing appearance to herbivores. Localized ethylene emissions following pollination usually trigger flower senescence and abscission. The lack of ethylene perception is one explanation for the observed “permaflower” of ethylene-insensitive 35S-etr1b plants (*Manuscript III*; Figure 3).

3.2.3. Signal crosstalk: the key to specificity

One individual signaling pathway is often not sufficient to explain patterns in the transcriptome, proteome, or metabolome of a plant observed after the attack by different herbivores or in comparison of responses in local and systemic tissue (Kessler and Baldwin, 2004; Rojo *et al.*, 2003; Voelckel and Baldwin, 2004b, 2004a). Crosstalk between different signaling cascades can modify the response to a single hormone and thereby increase the response specificity. Several hormones interact during specific responses and a classical example is the elicitation of induced systemic resistance (ISR), mediated synergistically by JA and ethylene, vs. the SA-dependent activation of systemic acquired resistance (SAR) (Pieterse *et al.*, 1998). These two induced defense responses are specifically induced by non-pathogenic vs. pathogenic bacteria and show varying degrees of effectiveness towards different secondary pathogen infections (Ton *et al.*, 2002), highlighting the importance of specific signaling cascades and their interactions for the activation of specific plant responses. Comparable interactions of hormone signaling cascades have been observed in the regulation of direct and indirect defenses against herbivores.

Application of acetylsalicylate (aspirin) inhibited PI transcript accumulation in tomato plants suggesting an antagonism between SA- and JA signaling pathways (Peña-Cortes *et al.*, 1993). Whereas the herbivore-induced volatile emission of maize (Schmelz *et al.*, 2003) and PI activity in tomato plants (O'Donnell *et al.*, 1996) are synergistically influenced by JA and ethylene. Furthermore, both hormones, JA and ethylene, are necessary for enhanced phenolic synthesis and formation of traumatic resin ducts two important defense responses of conifer species (Hudgins and Franceschi, 2004). In *N. attenuata* ethylene is not involved in the elicitation of *M. sexta*-induced VOC emissions (Kahl *et al.*, 2000) but ethylene and JA co-regulate direct defenses, e.g. nicotine and PI activity (*Manuscript II and III*). JA and ethylene production in *N. attenuata* is elicited by the same *M. sexta* OS-derived elicitor; FACs (Halitschke *et al.*, 2001; *Manuscript III*). Furthermore, both signaling cascades are dependent on a specific protein kinase, which is homologous to salicylic acid-activated protein kinase (SIPK) in tobacco (Wu *et al.*, 2007)

Additional signaling events downstream in the JA and ethylene signal cascades are likely to mediated further integration of the response to the two hormone signals. The activation of transcription factors like ERF1 and MYC2 integrate JA and ethylene signaling in Arabidopsis (Lorenzo and Solano, 2005). The concomitant induction by the same elicitor and later crosstalk at the level of transcriptional activation are two mechanisms allowing plants to integrate signaling pathways and fine tune their response to perceived environmental cues.

The regulation of each others' biosynthesis is a further possibility of signal crosstalk and several synergistic and antagonistic effects of a plant hormone on the biosynthesis of a second have been reported. A classical example is the SA-mediated suppression of JA biosynthesis (Peña-Cortes *et al.*, 1993). In contrast to the antagonistic effect of SA on JA biosynthesis, JA and ethylene show synergistic effects on each others biosynthetic pathway. Wound-induced JA accumulation increased ACS gene expression in winter squash (*Cucurbita maxima*) (Watanabe *et al.*, 2001) and MeJA application induced the ethylene formation in the internodes of two conifer species *Pseudotsuga menziesii* and *Sequoiadendron giganteum* (Hudgins and Franceschi, 2004). On the other hand, ethylene treatment induced allene oxide synthase (AOS) activity and increased JA accumulation in *Arabidopsis thaliana*. This ethylene-dependent JA production paralleled the second JA burst following the initial JA accumulation of wounded Arabidopsis leaves, which proposed that ethylene facilitates wound-induced JA biosynthesis (Laudert and Weiler, 1998). A comparable induction of AOS by ethylene was observed in tomato (Sivasankar *et al.*, 2000). In marked contrast, herbivore-induced ethylene and JA biosynthesis are independently regulated and not affected by the production of the other hormone in *N. attenuata*. Herbivore-induced ethylene emission were similar in wild type (WT) plants and transgenic plants silenced in JA production and conversely, JA levels were similar in plants impaired in ethylene signaling in comparison to levels in WT plants (Caroline C. von Dahl and Ian T. Baldwin unpublished data). Therefore, the specific regulation of nicotine accumulation by JA and ethylene observed in *N. attenuata* (Kahl *et al.*, 2000; Winz and Baldwin, 2001); *Manuscript III*) is mediated by downstream signaling events involving the inhibition of a JA-mediated wound response by ethylene without a direct effect of the hormones on each others biosynthesis. Crosstalk with additional signaling cascades extends the responses elicited 'single-handedly' by ethylene itself.

3.2.4. Priming responses by altered sensitivity

Altered signal sensitivity of a whole plant or specific tissue will increase the diversity of the responses that could be triggered by an individual signal. Sensitivity could be altered either at the level of detection of the external cue eliciting a plant response or at the level of endogenous hormone sensing mediating the response in the plant. Increased sensitivity could be a mechanism facilitating priming of induced defense responses (Figure 2). The physiological state of "being primed" allows a plant to respond faster or to a greater extend to a biotic or abiotic

stress and this enhanced response might be caused by the increased sensitivity towards external or endogenous signaling compounds (Engelberth *et al.*, 2004). Priming of induced responses to herbivory by volatile compounds, which avoids the costs of mounting a complete defense response without the actual stress exposure, could be a favorable mechanism of plant-plant communication in an evolutionary perspective (*Manuscript V*). Indeed priming has been observed in two studies of plant-plant interactions in nature (Heil and Bueno, 2007; Kessler *et al.*, 2006).

Besides the direct involvement of ethylene in the activation of induced responses to herbivory (see above; *Manuscript II* and *III*) its volatile nature and phytohormone properties make ethylene a perfect candidate signal for priming. Two studies revealed a function of ethylene in the priming of induced resistance by intra- and inter-plant signaling. In the first study on maize, the induction of VOC emission by (*Z*)-3-hexen-1-ol was synergized by exposure to ethylene, and increased 2.5-fold in the presence of ethylene (Ruther and Kleier, 2005). However, exposure to ethylene alone elicited no response suggesting an enhanced sensitivity to (*Z*)-3-hexen-1-ol induced by ethylene (Ruther and Kleier, 2005). In the second study, feeding by *Pieris rapae* on *Arabidopsis thaliana* plants not only triggered a systemic resistance against subsequent herbivory, but also increased resistance against the Turnip crinkle virus (TCV) (de Vos *et al.*, 2006). In these experiments herbivore-induced ethylene increased the sensitivity of *Arabidopsis* leaves to SA and augmented the expression of SA-dependent defense genes such as *PR-1* in the primed leaves (de Vos *et al.*, 2006). The transgenic plants generated and characterized in this study (*Manuscript III*) represent valuable tools to further dissect the response in *N. attenuata* plants exposed to VOCs released *M. sexta*-damaged conspecifics (*Manuscript V*).

In addition to the priming effect of ethylene itself, the characteristics of ethylene reception would make ethylene sensing an ideal priming target. The negative regulation of ethylene signaling which is dependent on the proportion of receptors bound to ethylene molecules allows a plant to down-regulate ethylene sensitivity by increased receptor production and increase its sensitivity with a reduced abundance of ethylene receptors (Klee, 2004). This regulation of ethylene sensitivity is a major regulatory component of fruit ripening in tomato (Klee, 2004) and increased ethylene sensitivity could represent a priming mechanism in the context of induced defenses.

3.3. Conclusion

The volatile signals MeOH and ethylene are signals involved in the regulation of plastic responses, such as the gain of immunity or resistance against insect attack (*Manuscript I* to *III*) and the presumably mutualistic interactions with pollinating insects, growth-promoting fungi, and other plants during plant-plant communication (*Manuscript III* to *V*). Phenotypic plasticity is a means to increase fitness as the local environment fluctuates (Silvertown, 1998) and indeed an ethylene-dependent fitness benefit has been observed in *N. attenuata*. Using the ethylene

perception blocker 1-methylcyclopropen (1-MCP) ethylene signaling was excluded from the *M. sexta* larvae-*N. attenuata* interaction and the performance of *N. attenuata* plants decreased (Voelckel *et al.*, 2001). This could be attributed to the reduced accumulation of nicotine in the presence of ethylene, a potent defense that is costly for the plant but less effective against the specialist attacker *M. sexta* (*Manuscript III*). Additionally, increased fitness of *N. attenuata* plants due to the regulation of ethylene signaling is suggested during the growth-promoting association with *S. vermifera* (*Manuscript IV*), and in the termination of floral display after fertilization (*Manuscript III*). On the contrary, the production of MeOH, a product of PME activity, is likely to impose fitness costs on *N. attenuata* plants, not because of the energy demanding biosynthesis pathway, but because *M. sexta* larvae fed on MeOH exposed plants performed better than larvae fed on water-exposed control plants (*Manuscript I*). Whether or not the induced MeOH emission is beneficial for the plant due to other regulated responses will be investigated using ir-*pme* plants silenced in *NaPME* transcription and impaired in herbivore-induced MeOH production.

4. Summary

To succeed in a fluctuating environment, plants have to adjust their phenotype continuously so that the present environment resembles a favorable habitat. Direct and indirect defenses are rapid and plastic responses involved in herbivore resistance and are mainly regulated by the phytohormone jasmonic acid (JA) (Halitschke and Baldwin, 2003). However, plant defenses against herbivory are not solely regulated by JA.

In this study the volatile compounds methanol (MeOH) and ethylene were characterized with regard to their biosynthetic regulation and signaling function in the defense response of *Nicotiana attenuata* plants under attack by *Manduca sexta* larvae. Herbivore-induced MeOH emissions increased above wound-induced emissions. This phenomenon could be attributed to the alkaline pH of the hornworm oral secretions (OS). The OS-induced pectin methylesterase (PME) activity, which is paralleled by reduced methylation of pectin molecules after OS elicitation and followed by higher transcript accumulation of *NaPME*, demonstrates PME-mediated pectin demethylation as the source of herbivore-induced MeOH emissions. In contrast, herbivore-induced ethylene emissions were triggered by the OS-derived elicitor fatty-acid amino-acid conjugates (FACs), which is also responsible for the elicitation of herbivore-induced JA-biosynthesis. Ethylene emissions are regulated through the transcript accumulation of *NaACS3a*, *NaACO2a* and *NaACO3* and partially terminated by feedback regulation through ethylene perception.

A signaling function for MeOH was suggested by pharmacological experiments in which caterpillars fed on plants previously exposed to MeOH performed better than caterpillars fed on water-treated control plants. Ethylene's signaling function has previously been described as an ethylene-dependent attenuation of the JA-induced nicotine biosynthesis in response to the feeding of specialist *M. sexta* larvae on *N. attenuata* plants (Kahl *et al.*, 2000). The increased herbivore-induced nicotine accumulation of transgenic *N. attenuata* plants impaired in ethylene biosynthesis and perception, and the summary of the current literature on ethylene in herbivore-plant interactions confirmed ethylene's modifier function in the activation of various herbivore-induced defenses. The role of ethylene as a mediator of phenotypic plasticity was further suggested by the association of *N. attenuata* plants with *Sebacina vermifera*, in which reduced ethylene biosynthesis resulted in increased growth of *N. attenuata* plants associated with *S. vermifera*.

The presented study describes the signaling function of MeOH and ethylene in response to herbivory and fungus-association and discusses the challenges for scientists to identify new volatile signals or to relate new functions to known signals. Pinpointing signals becomes specifically difficult when a signaling function within a plant is to be extended to a signaling function between plants in plant-plant communication.

5. Zusammenfassung (German)

Pflanzen sind trotz ihrer sessilen Lebensweise weitaus aktiver als es den Anschein hat. In einem sich ständig verändernden Lebensraum passen Pflanzen kontinuierlich ihren Phänotyp den herrschenden Umweltbedingungen an. Um auf abiotische und biotische Stressfaktoren reagieren zu können, haben Pflanzen Erkennungs- und Regelungsmechanismen entwickelt, die es ihnen erlauben, gezielt und schnell auf momentan wirkende Umweltfaktoren zu reagieren. Mechanische Verwundung stellt dabei einen Reiz dar, der sowohl durch die unbelebte als auch durch die belebte Umwelt ausgelöst werden kann. Für die Pflanze ist es relevant diese voneinander zu unterscheiden. Zellwandbestandteile von Pilzen und Bakterien oder spezifische Abbauprodukte der Verdauungsenzyme angreifender Insekten sind für biotische Angreifer charakteristische Signalmoleküle, die die Pflanze erkennt und daraufhin eine spezifische, je nach Stressor unterschiedliche, induzierte Abwehrreaktion auslöst. Die Aktivierung bestimmter Abwehrmechanismen gegen Herbivorie, wie die Akkumulation toxischer Substanzen oder die Aktivierung verdauungshemmender Enzyme, erfolgt hauptsächlich über das Pflanzenhormon Jasmonsäure (JS) und dessen Derivate. Die Regulation der Abwehr findet jedoch nicht ausschliesslich über JS statt. Besonders die Vernetzung verschiedener Signalkaskaden ermöglicht der Pflanze eine spezielle Anpassung ihrer Reaktion auf die einwirkenden Reize.

Zielsetzung der vorgelegten Arbeit war die Bestimmung der Signalfunktion der beiden gasförmigen Substanzen Methanol und Ethylen in der herbivoreninduzierten Abwehrreaktion des wilden Tabaks (*Nicotiana attenuata*). Gegenstand der Untersuchung war die spezifische Regulation der Biosynthese und die Rolle der beiden Substanzen während der Interaktionen von *N. attenuata* mit Artgenossen, unter Herbivorie und in Wurzelsymbiose. Aus den Untersuchungsergebnissen lassen sich die folgenden Thesen ableiten:

Methanol zeigt Signalfunktionen in der Raupen-Wirtspflanzen Interaktion.

- Verwundung eines Blattes erhöht die Methanolemissionen von *N. attenuata*, die durch die oralen Sekrete (OS) von Larven des Tabakschwärmers (*Manduca sexta*) verstärkt werden. Der alkalische pH-Wert der OS lepidopterer Larven verusacht die erhöhte Methanolemissionen.
- Die induzierte Methanolproduktion korreliert mit erhöhter Aktivität der Pektinmethylesterase (PME) und einer damit einhergehenden verminderten Methylierung des Pektins. PME-Aktivität ist notwendig für die verstärkten Methanolemissionen während Schädlingsbefall.
- Die gesteigerten Methanolemissionen während der ersten 30 Minuten nach OS-Behandlung sind nicht transkriptionell reguliert; eine verstärkte Genexpression von *NaPME* erfolgt später.
- Methanol besitzt eine Signalfunktion in der untersuchten Raupen-Wirtspflanzen-Interaktion und führt durch eine verringerte Aktivität verdauungshemmender Enzyme zu einer verbesserten

Entwicklung der Larven des Tabakschwärmers auf methanolexponiertem wilden Tabak im Vergleich zu wasserexponierten Pflanzen.

Ethylen reguliert die induzierte Schädlingsabwehr durch die Modifizierung JS-abhängiger Abwehrreaktionen.

- Die durch Schädlingsbefall induzierte Ethylenbiosynthese ist unabhängig von der durch Verwundung oder Pathogene verursachten Ethylenemission.
- Neun Abwehrmechanismen gegen Herbivorie sind ethylenabhängig reguliert; davon wurden acht in pharmakologischen Studien beschrieben und nur eine bei Untersuchungen transgener Pflanzen ermittelt.
- Die ethylenbedingte Regulation vieler Abwehrmechanismen ist primär von anderen Signalen wie z.B. JS abhängig.
- Die Verwendung transgener Pflanzen zur Untersuchung der Vernetzung von Signalkaskaden ist aussagekräftiger als pharmakologische Studien.

Die OS-induzierte Ethylenbiosynthese in *N. attenuata* ist transkriptionell und über eine negative Rückkopplung reguliert.

- Fettsäure-Aminosäure-Konjugate in den OS der *M. sexta* Larven sind die auslösenden Signale für die herbivorenspezifisch induzierte Ethylenemission.
- Die virusbasierte Reduktion der Genexpression von ACC Synthase (ACS) und ACC Oxidase (ACO) konnte in Verbindung mit pharmakologischen Studien zeigen, dass die ACS Aktivität der geschwindigkeitsbestimmende Schritt der OS-induzierten Ethylenbiosynthese ist und dass die Genexpression von *NaACO2a* und *NaACO3* für die OS-induzierte Ethylenemission relevant ist.
- Die Ethylenbiosynthese ist transkriptionell reguliert. Die Genexpression von *NaACS3a* und *NaACO2a* wird spezifisch durch OS aktiviert, während die Transkription von *NaACO1* nach mechanischer Verwundung ansteigt und durch OS erhöht wird. Die Akkumulation von *NaACO3* mRNA ist induziert durch Verwundung und zeigt keine herbivorenspezifische Regulation.
- Eine negative Rückkopplung limitiert die maximale Ethylenemission. Pflanzen mit reduzierter Ethylensensitivität zeigen eine erhöhte Ethylenemission nach OS Behandlung.
- Ethylen hemmt die OS-induzierte Akkumulation von Nikotin und fördert die Abszission von bestäubten Blüten. Pflanzen mit reduzierter Ethylenbiosynthese und reduzierter Ethylensensitivität akkumulieren mehr Nikotin nach OS-Induktion und zeigen eine verlängerte Blütezeit.

Die Wachstumsföderung von *N. attenuata* durch den Pilz *Sebacina vermifera* beruht auf der verringerten Ethylenbiosynthese in pilzassoziierten Pflanzen.

- In Wurzelsymbiose mit *S. vermifera* wächst *N. attenuata*, unabhängig von JS und deren Derivaten und durch sie regulierter Verteidigungsmechanismen, grösser und bildet mehr Samenkapseln.
- Transgene Pflanzen mit verringerter Ethylenbiosynthese zeigen keinen positiven Wachstumseffekt in Assoziation mit *S. vermifera*. Wildtyp Pflanzen in Assoziation mit *S. vermifera* zeigen eine teilweise durch reduzierte Genexpression bedingte, reduzierte Ethylenbiosynthese.
- Sämlingsstudien mit verringerter oder erhöhter Ethylenexposition bestätigen, dass *S. vermifera* die Ethylenbiosynthese inhibiert und dadurch das Wachstum der Pflanzen verbessert.

Gasförmige Substanzen sind mögliche Signale der Kommunikation zwischen Pflanzen.

- Mehrere Studien zeigten eine erhöhte Abwehrreaktion gegen Schädlingsbefall in Pflanzen, die bestimmten von attackierten Pflanzen emittierten Duftstoffen ausgesetzt waren. Getestete Substanzklassen waren Grünblattalkohole, Ethylen, Hormonderivate wie Methyljasmonat und Methylsalicylat, sowie Terpene.
- Gasförmige Substanzen mit Signalfunktion innerhalb einer Pflanze sind besonders dazu geeignet auch als Signale zwischen Pflanzen zu dienen.
- Die Untersuchung transgener Pflanzen in ihrem natürlichen Habitat erlaubt die Identifikation der aktiven Signalstoffe unter ökologisch relevanten Bedingungenen.

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8. Declaration of Independent Assignment

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of Friedrich Schiller University Jena that the submitted thesis was written only with the assistance and literature cited in the text.

People who assisted in the experiments, data analysis, and writing of the manuscript are listed as coauthors of the respective manuscripts. I was not assisted by a consultant for doctorate theses.

The thesis has not been previously submitted either to the Friedrich-Schiller-University Jena or to any other University.

Jena, July 4th 2007 _____

Caroline C. von Dahl

9. Curriculum vitae

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Aug. 1986-July 1995	High School Diploma, St. Lioba Gymnasium, Bad Nauheim
Aug. 1982-July 1986	Karl-Weigand Schule, Florstadt
Professional and Scientific Career	
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June 2001-July 2002	Diploma thesis at Max Planck Institute for Chemical Ecology, Department of Molecular Ecology, Jena; “Das Ende der Octadecanoid-Kaskade: Untersuchung der Funktionen der Jasmonsäurederivate Jasmonsäuremethylester und cis-Jasmon in *Nicotiana attenuata*”
June 2000	Student assistant at University Hamburg, Institute of Geography
Feb. 2000-March 2000	Volunteer at University of New South Wales, Department of Biological Sciences, Prof. Barry J. Fox, Australia; “Modeling of the assembly rule for community structure”
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Publications

Barazani, O., von Dahl C.C., & Baldwin I.T. (2007) *Sebacina vermifera* promotes the growth and fitness of *Nicotiana attenuata* by inhibiting ethylene signaling. *Plant Physiol.* **144**, 1223-1232

von Dahl C.C., Winz R.A., Halitschke R., Kühnemann F., Gase K., & Baldwin I.T. (2007) Tuning the herbivore-induced ethylene burst: the role of transcript accumulation and ethylene perception in *Nicotiana attenuata*. *Plant J.* **51**, 293-307

von Dahl C.C. & Baldwin I.T. (2007) Deciphering the role of ethylene in plant-herbivore interactions. *J. Plant Growth Regul.*, **early online**

von Dahl C.C., Hävecker M., Schlögl R., & Baldwin I.T. (2006) Caterpillar-elicited methanol: a new signal in plant-herbivore interactions? *Plant J.* **46**, 948-960

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von Dahl C.C. & Baldwin I.T. (2004) Methyl jasmonate and *cis*-jasmone do not dispose of the herbivore-induced jasmonate burst in *Nicotiana attenuata*. *Physiol. Plant.* **120**, 474-481

Conference Contributions

von Dahl C.C. & Baldwin I.T. (2007) Herbivore induced emission of small-molecular-mass compounds: waste product, artifact, or active signal? *EPS symposium on "Ecology and Experimental Plant Sciences: From Molecules to Multitrophic Interactions"*, Wageningen, **invited talk**

von Dahl C.C. & Baldwin I.T. (2007) Tuning the defense response with VOCs: methanol and ethylene in plant-insect interactions. *PR-IR workshop*, Doorn, **invited talk**

von Dahl C.C. & Baldwin I.T. (2006) Herbivore induced ethylene and its impact on *Nicotiana attenuata*'s defense response. *7th International Symposium on the Plant Hormone Ethylene*, Pisa, **talk**

von Dahl C.C. & Baldwin I.T. (2002) Methyl jasmonate and *cis*-jasmone: inactivation or amplification of the octadecanoid pathway? *19th Annual Meeting of the International Society of Chemical Ecology*, Hamburg, **poster**

10. Supplementary Material

10.1. Manuscript I

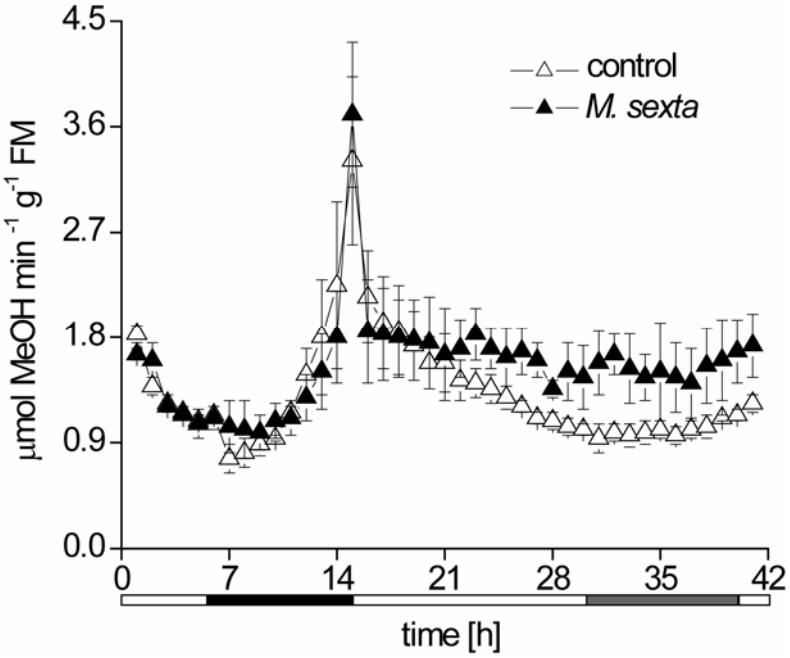

Figure S1. Pattern of MeOH emission from attacked and control *N. attenuata* plants over 40 h of continuous measurements.

Mean µmol MeOH min^{-1} g^{-1} FM ± SE released by control plants (open symbol) and plants that were under attack by 5 neonate *M. sexta* larvae (filled symbol). MeOH emission was analyzed by proton-transfer-reaction mass spectrometry (PTR-MS) configured with a six-port valve (Figure 1a), which sampled the headspace for 10 min of each of 6 individual plant chambers for 40 h. Open and dark bars designate day and night periods, respectively. The two night cycles differed in their light regime indicated by the black (lights off) and gray (lights on) bar. Note the high rates of MeOH emission from both attacked and control plants at the beginning of the light phase.

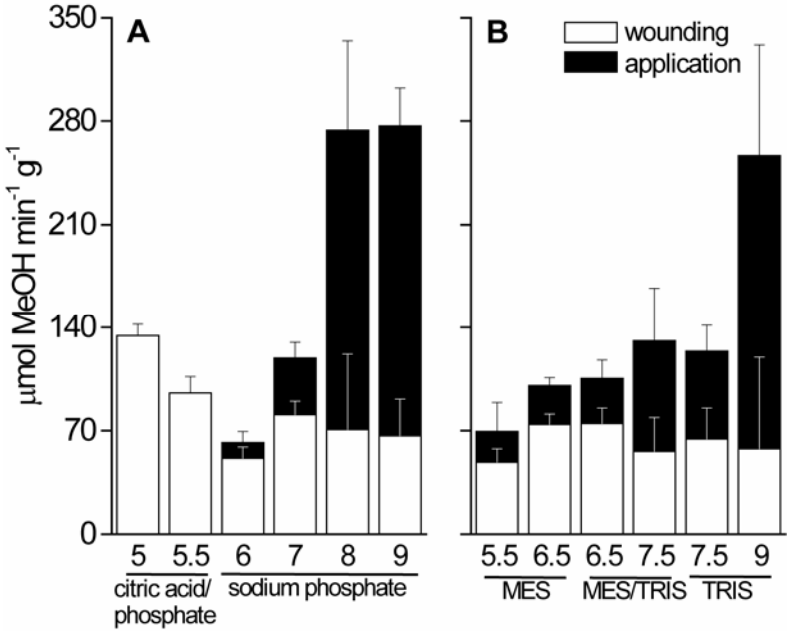

Figure S2. Induced MeOH emissions increase with increasing pH.
Mean µmol MeOH min^{-1} g^{-1} FM ± SE released from single *N. attenuata* leaves after standardized puncture wounding and application to the wounds of 20 µl of **A**. 0.1 M phosphate buffers with the indicated pH values and **B.** 0.1 M MES, 0.1 M MES/TRIS, and 0.1 M TRIS with the indicated pH values. Wound-induced MeOH emission (white bars) and MeOH emissions elicited after the test solutions were applied to the puncture wounds (black bars) are presented.

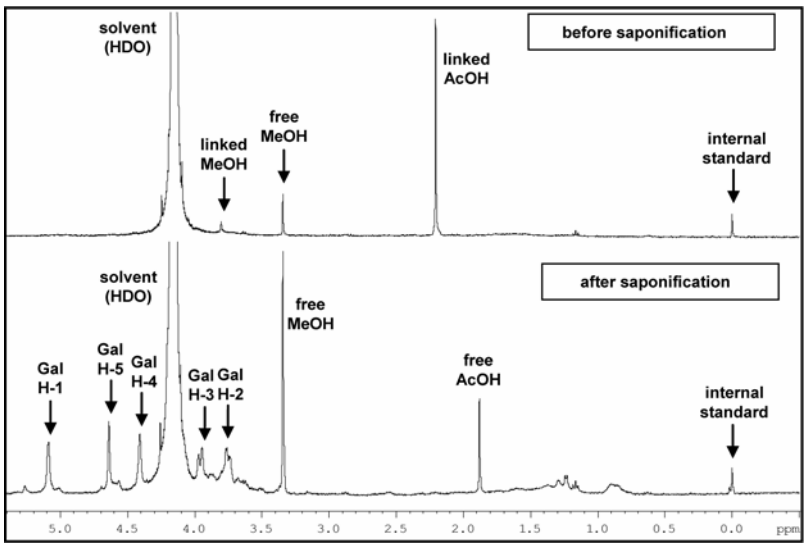

Figure S3. ^{1}H NMR spectra of pectin extracts before and after saponification.

NMR measurement was performed on a Bruker DRX 400 NMR spectrometer, operating at 500, 13 MHz for ^{1}H. The samples were measured in D_4-MeOH. Peaks are labeled according to their representative chemical shifts given in δ values in reference to a TMS as internal standard with coupling constants in Hz. An example of a wounded leaf is presented. After the first measurement at 80°C, NaOD was added to the NMR tube and further scans were recorded to measure the loss of methylesters upon saponification. Note that the signals of esterified MeOH and AcOH disappear after saponification and that the free MeOH increases.

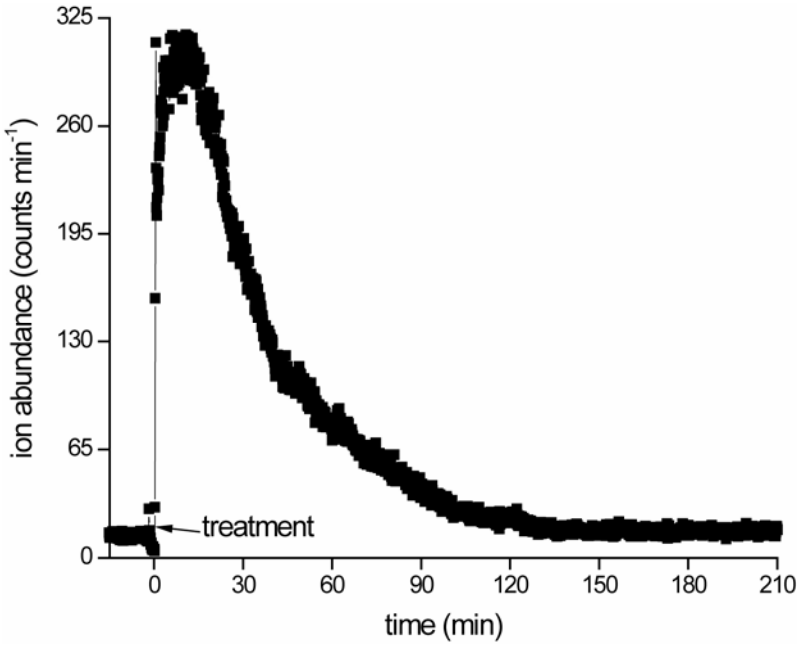

Figure S4. A representative PTR-MS scan of MeOH after wounding and OS application.

Relative MeOH concentration (counts second^{-1} of m/s 33) in the emission of a single leaf. The emission of an untreated control leaf is shown before time = 0 min, the time of treatment. The induced MeOH emission, by wounding, and application of 20 µl of OS of *Manduca sexta* (1:1 diluted with water) reached a maximum about 15 min after the treatment and returned to control levels at about 120 min.

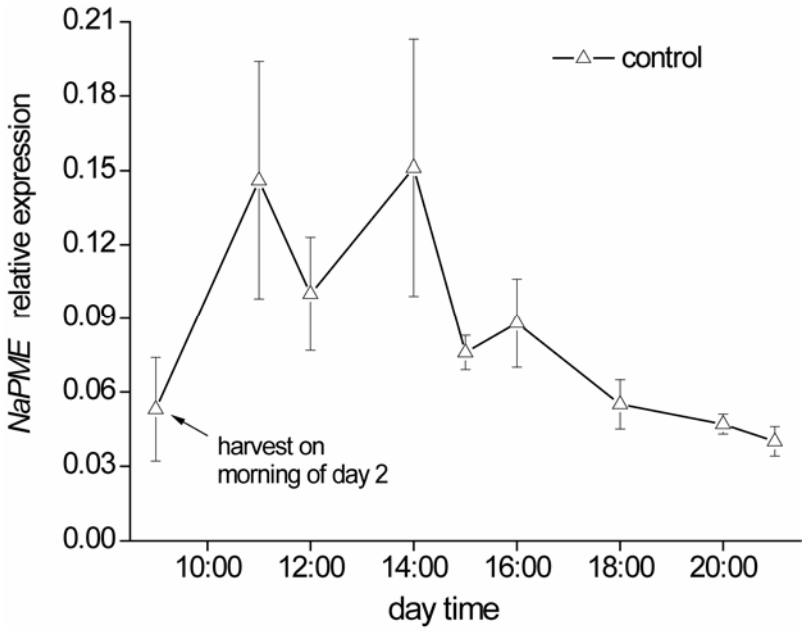

Figure S5. Diurnal expression of *NaPME* in *N. attenuata*.

Relative expression ratio ± SE in arbitrary units of *NaPME* transcripts analyzed by RT-PCR. cDNA was transcribed from 5 replicated untreated *N. attenuata* plants (open triangles) for each time point. Samples were harvested over a 24-h period, with the samples at 9:00 am harvested on the second day. The light/dark regime was set to 16 h:8 h starting at 6:00 am.

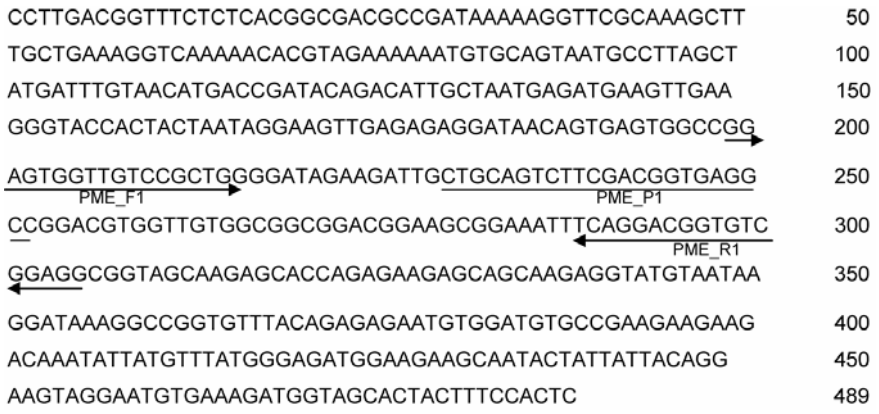

Figure S6. *Nicotiana attenuata* partial mRNA for pectin methylesterase
Sequence information on *NaPME* (Accession No. DQ115979). Forward and reverse primers (PME_F1 and PME_R1) and probe (PME_P1) used for RT-PCR assays are underlined.

10.2. Manuscript III

Table S1. *ACS* and *ACO* gene families are highly conserved in Solanaceous plants. Percent sequence identity of pair wise comparison between *ACS* (**A**) and *ACO* (**B**) mRNA of *N. attenuata* and other *Solanaceae.*

		N. attenuata		
A		*ACS1*	*ACS2*	*ACS3a*
N. tabacum	ACS (X65982)	94	71	72
	ACCS2 (X98492)	71	93	81
	ACS2 (AJ131836)	73	80	95
N. glutinosa	ACS1 (AF057562)	94	70	71
	ACS (AF061605)	73	82	83
	ACS2 (AF057563)	73	81	94
S. lycopersicum	ACC2 (X59145)	85	76	72
	ACS6 (AF179249)	74	80	80
	ACS1a (U72389)	74	77	86

B		*ACO1*	*ACO2a*	*ACO3*
N. tabacum	EFE (Z29529)	95	89	79
	ACO (AB012857)	88	93	78
	ACO (X83229)	79	78	97
N. glutinosa	ACO1 (U54565)	88	89	79
	ACO3 (U62764)	76	79	81
	ACO2 (U54566)	77	78	94
S. lycopersicum	EFE (X58885)	85	86	78
	ACO1 (X58273) gene	86	90	83
	ACO4 (AB013101)	78	80	83

Table S2. Primer selection for gene-specific probes of *NaACS, NaACO*, and *ETR1*.

Gene	Template accession #	Primer/probe	Sequence	Amplicon [bp]
ACS library screening				
NtACS1	Q07262	NtACS1F	GGGATTTGAGAATGAGAA	1451
		NtACS1R	GTGAATGAGGGATAGGAG	
NtACS2	CAA67118	ACSNt2F	ACAATGAGCTGCTTTCTA	1305
		ACSNt2R	CTCCTTCAATCCCTTTAC	
ACO library screening				
NtACO1	CAA67119	ACONtF	GGGGCTTCTTTGAGTTGG	726
		ACONtR	TCTCCGCTGCCTCTTTCT	
N. attenuata ACS gene-specific hybridization				
NaACS1	AY426752	NaACS1-3	TCTCGCCTGGATCTTCAT	366
		NaACS1-1	GAATCATTCTACTACCTTCTTCTT	
NaACS2	AY426753	NaACS2-2	AGAGCCTGGTTGGTTTAG	306
		NaACS2-1	CATTCAATTTCGAAGAATTATC	
NaACS3a	AY426754	NaACS3a-7	ATTCTCCTTTGGTCAGGA	193
		NaACS3a-4	ACTCCAAACTTACTTGTTTATTT	
NaACS3b	AY426755	NaACS3b-6	ACCTTTGGTCAGGACTTG	185
		NaACS3b-3	TCTCTGAAGAACTTGTTTCTTAT	
N. attenuata ACO gene-specific hybridization				
NaACO1	AY426756	NaACO1-2	GTGATCTATCCCGCACCA	305
		NaACO1-1	AAATATATTAATTCCACTATACTAAAC	
NaACO2a	EF123109	NaACO2a-4	CAAGGTTTGAAGCCATGA	194
		NaACO2a-1	GAATAGCTTGATTCCACCA	
NaACO2b	EF123110	NaACO2b-4	GGTTTGAAGCCATGATCA	175
		NaACO2b-1	GTATAATTCTACGATACTAAAGAAGC	
NaACO3	EF123111	NaACO3-2	GTCATCTATCCAGCACCA	294
		NaACO3-1	AAACACCCCCACTAATCA	
N. attenuata ETR1 and Arabidopsis etr1-1 gene-specific hybridization				
NaETR1	EF203416	NaETR1_for	GTTTGAGAGATGCAGAAGCG	398
		NaETR1_rev	GCTGCACAGCTTGATCGTG	
35s-etr1a	AC020665	etr1_for	GAAAGGTGGAGAAGCGGAAC	397
		etr1_rev	GCTGCTGAGCTCGATAGAG	
35setr1b/c	pCD2	ETR2-3_for	GCTAGAGTCAGCTTGTCAG	360
	NM_105305	ETR2-3_rev	CAAAGCAAGTGGATTGATGTG	
Transgene insertion screening for hygromycine resitance gene				
hptII	pCAMBIA1301	Hyg1-18	CGTCTGTCGAGAAGTTTCTG	873
	AF234297	Hyg3-20	CCGGATCGGACGATTGCG-5	
N. attenuata gene-specific quantitative real time PCR				
NaETR1	EF203416	NaETR F1	TGACTGCGCTGGTATCATGTA	
		NaETR R1	TTTCACGATCAAGCTGTGCA	
		NaETR_P1	FAM-AACTGCGCTCATGCTTGTCCACATT-TAMRA	
NaACO1	AY426756	TM_NaACO1F6	CTATTGAATCTGATGTCAAGCTG	
		TM_NaACO1R6	TATGTAGTAGGGACACACGCTT	
		NaACO1_P	FAM-CAACTGCATAGATCCAAATTCAAGAGTACTAAAG-TAMRA	
NaACO2a	EF123109	TMaco2-F3	CCATGGAAACTGGTGTGAAATG	
		TMaco2-R3	AGCTTGATTCCACCACACACAA	
		TM_ACO2-P	FAM-CAACTGCTTAAATTCCAATTCGAGAGGAAGGA-TAMRA	
NaACO3	EF123111	TM-ACO3F	AAGCAATGAAGGCTGTGGAAA	
		TM-ACO3R	ACCCAAGTGGCATAAAACAAGAA	
		TM-ACO3P	FAM-CAACTCTGCCCCAATAGCAACTGTTTGAGA-TAMRA	
NaACS3a	AY426754	TM_ACS3_F1	ATCCTTCAAATCCATTAGGCAC	
		TM_ACS3_R1	AACACTGATGAATTTCGGCTG	
		NaACS3_P	FAM-TGCGACGAAATTTATGCTGCTACTGTC-TAMRA	

Table S3. Primer selection of plasmid design for transformation.

Construct	Template accession #	Primer	Sequence
pRESC5ACO1	AY426756	ACOR1	GCGGCGCCATGGAGATCACTGCATCACTTCCTGG
		ACOF1	GCGGCGCTGCAGGTGGCATAATCCTTCTCTTCC
		ACOR2	GCGGCGCTCGAGAGATCACTGCATCACTTCCTGG
		ACOF2	GCGGCGGAGCTCGGTGGCATAATCCTTCTCTTCC
pTVaco1	AY426756	ACO1 1-32	GCGGCGGTCGACGATCTATCCCGCACCAGCTC
		ACO1 2-32	GCGGCGGGATCCGATTCAATAGATTTCATAGC
PTVaco2	EF123109	ACO2 1-31	GCGGCGGTCGACGAAGTTATATGCTGGACTC
		ACO2 2-31	GCGGCGGGATCCCAATAAGATCACATTTCAG
pTVaco3	EF123111	ACO3 1-31	GCGGCGGTCGACGTCATCTATCCAGCACCAG
		ACO3 2-32	GCGGCGGGATCCCTTCATTGCTTCAAACCTTG
pTVacs	AY426754	ACS1-34	GCGGCGGTCGACATGGACATGGAGAAAACTCAGC
		ACS2-31	GCGGCGGGATCCTCATTACTATGCGATTTGG

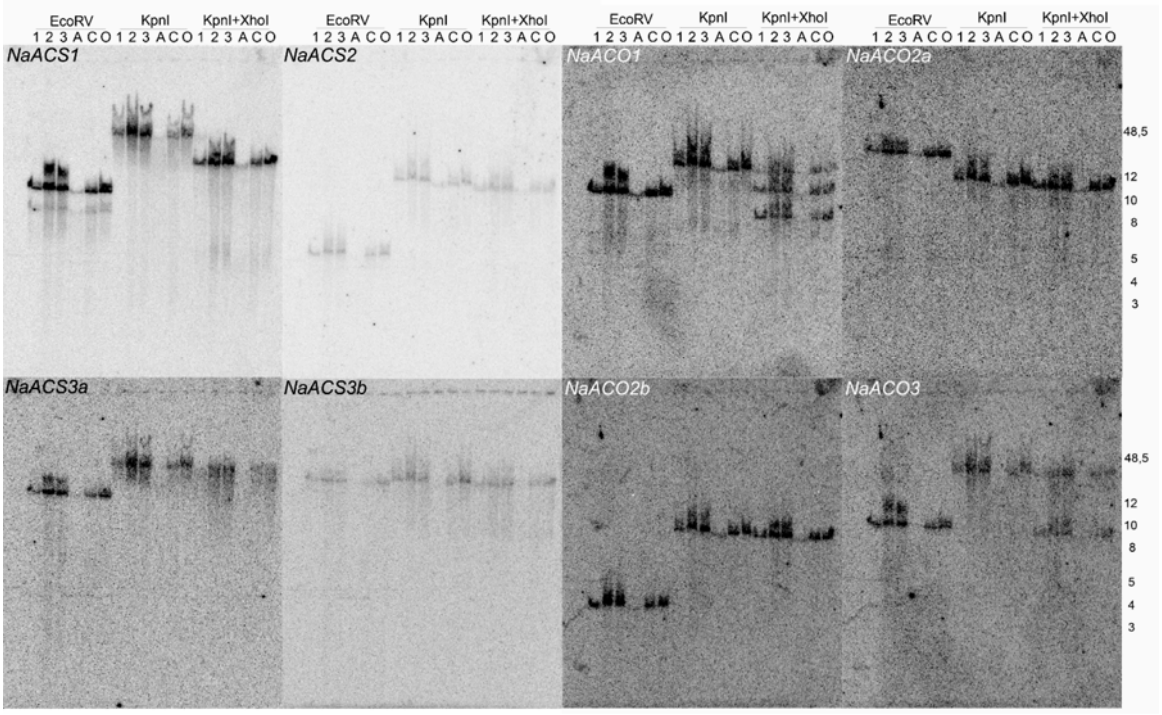

Figure S1. Southern blot analysis of *N. attenuata ACS* and *ACO* genes.

Genomic DNA of plants from six populations of *N. attenuata* collected in the Great Basin desert of Utah (1, 2, and 3), Arizona (A), California (C), and Oregon (O), was digested with EcoRI, KpnI, and KpnI+XhoI, blotted onto a nylon membrane and hybridized with gene-specific probes. No restriction sites for the selected enzymes are found in the exon 4 region of all genes, to which the labeled gene-specific probes hybridize. DNA marker size (kb) is indicated.

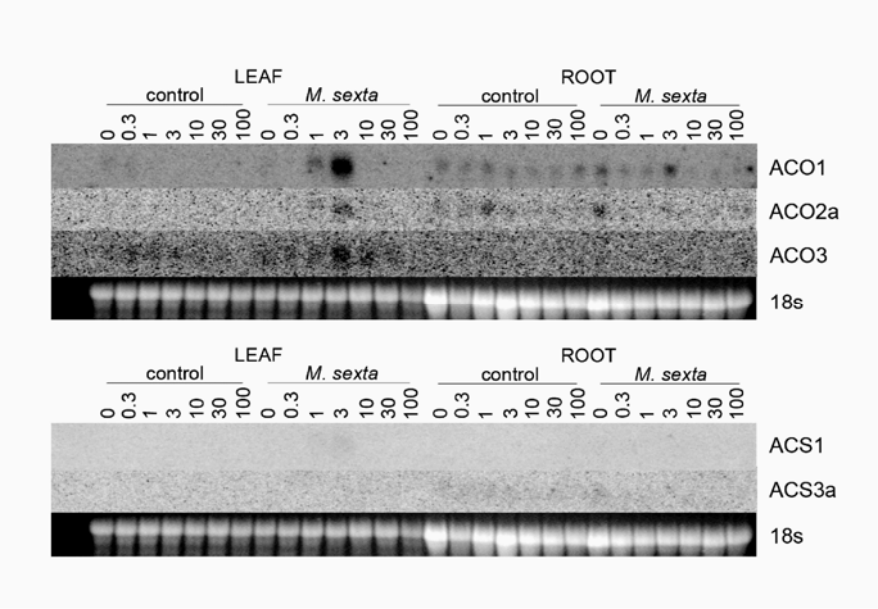

Figure S2. *M. sexta*-induced transcript accumulation of *ACO* and *ACS* genes.
Total RNA (10 µg) extracted from leaves and roots of individual *N. attenuata* plants harvested after damage by three *M. sexta* larvae. Larvae were allowed to feed for 1 h and tissue was harvested at the indicated times (h). Control plants remained untreated. Blots were hybridized with specific probes for *NaACO1*, *NaACO2a*, *NaACO3*, *NaACS1*, or *NaACS3a*. Ethidium bromide stained 18S rRNA is shown as loading control.

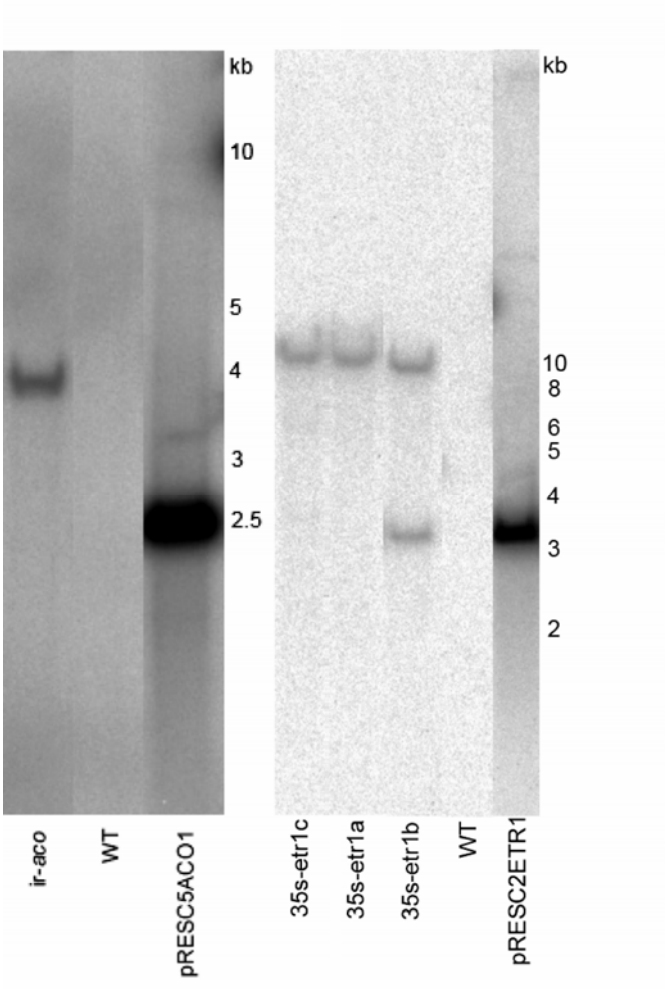

Figure S3. Number of transgene insertions of ir-*aco* and 35s-etr1 lines. Southern hybridization of genomic DNA extracted from *N. attenuata* WT and A03-321-10 (ir-*aco*) A03-318-10 (35s-etr1c), A03-328-8 (35s-etr1a), and A03-538-1 (35s-etr1b) plants. 10 µg of genomic DNA or transgene-containing plasmids (pRESC5ACO1, pRESC2ETR1) were digested with EcoRV. The digest was separated on an agarose gel, blotted onto nylon membranes and hybridized with a ^{32}P-labelled *hptII*-specific probe. DNA marker size (kb) is indicated.

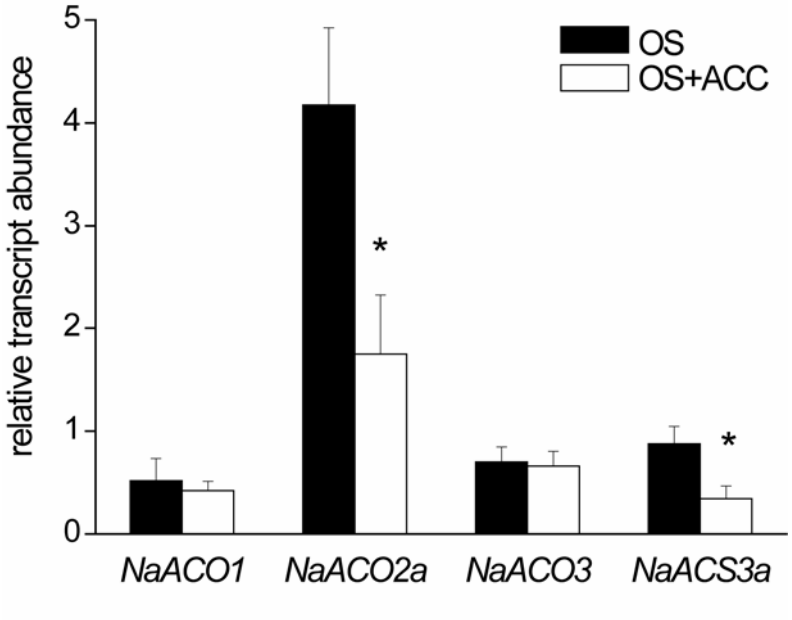

Figure S4. ACC-induced transcript accumulation in EV inoculated *N. attenuata* plants.

Relative transcript abundance (mean ± SE, n = 5) of ACO and ACS genes was analyzed by RT-PCR in EV inoculated WT plants after elicitation with OS and OS supplemented with 5 mM ACC. *NaACO1* and *NaACO3* transcripts were analyzed 3 h after elicitation, while transcripts of *NaACO2a* and *NaACS3a* were measured 1 h after the treatment. Transcript accumulation was normalized to the transcript levels of ECI which are not regulated by OS-elicitation. Asterisks represent significant differences between the treatments (Students *t*-test, $P < 0.05$).

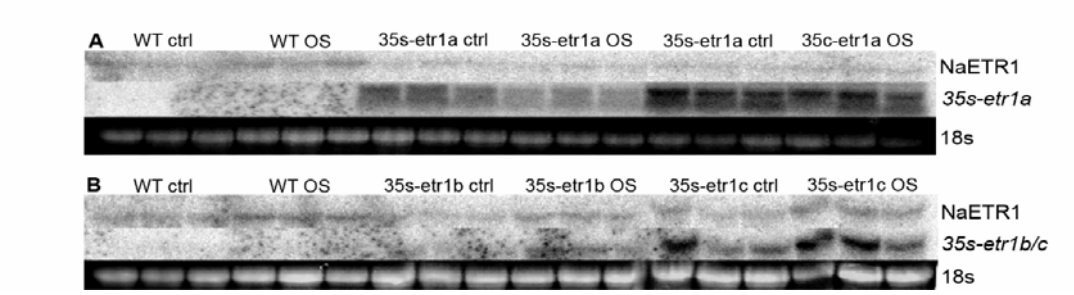

Figure S5. Endogenous and heterologous expression of *ETR1* in 35s-etr1 lines.
Total RNA (10 □g) of leaves of 3 individual *N. attenuata* plants harvested 10 h after elicitation by wounding and the application of *M. sexta* oral secretions (OS). Control samples were harvested of untreated plants (ctrl). RNA was blotted onto a nylon membrane and hybridized with specific probes. **A.** RNA of WT and 35s-etr1a (represented by two independently transformed lines) plants was hybridized with specific probes for *NaETR1* and the 35s-etr1a construct. **B.** RNA of WT, 35s-etr1b, and 35s-etr1c plants was hybridized with specific probes for *NaETR1* and the 35s-etr1b/c construct.

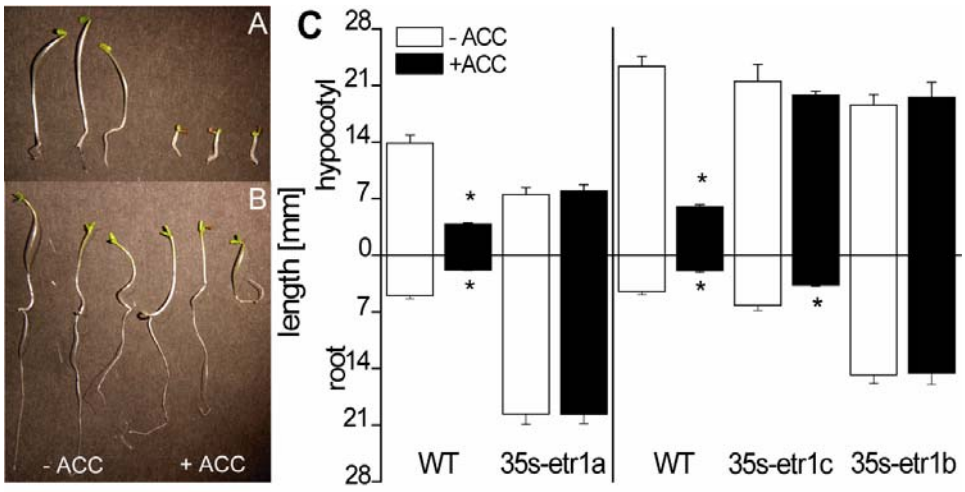

Figure S6. Triple response assay of *N. attenuata etr*-lines.

N. attenuata plants expressing the mutant *etr1-1* ethylene receptor from *Arabidopsis thaliana* under the control of a 35S promoter were tested for ethylene sensitivity. Inhibition of root and shoot elongation was analyzed in etiolated **A.** WT and **B.** 35s-etr1a *N. attenuata* seedlings in response to 5 µM ACC in the growth media. **C.** Root and shoot length of etiolated WT, 35s-etr1a, 35s-etr1b, and 35s-etr1c seedlings was determined in the presence (filled bars) and absence (open bars) of ACC in the germination media. Asterisks indicate significant length differences between seedlings grown with and without ACC (Students *t*-test, $P < 0.05$).

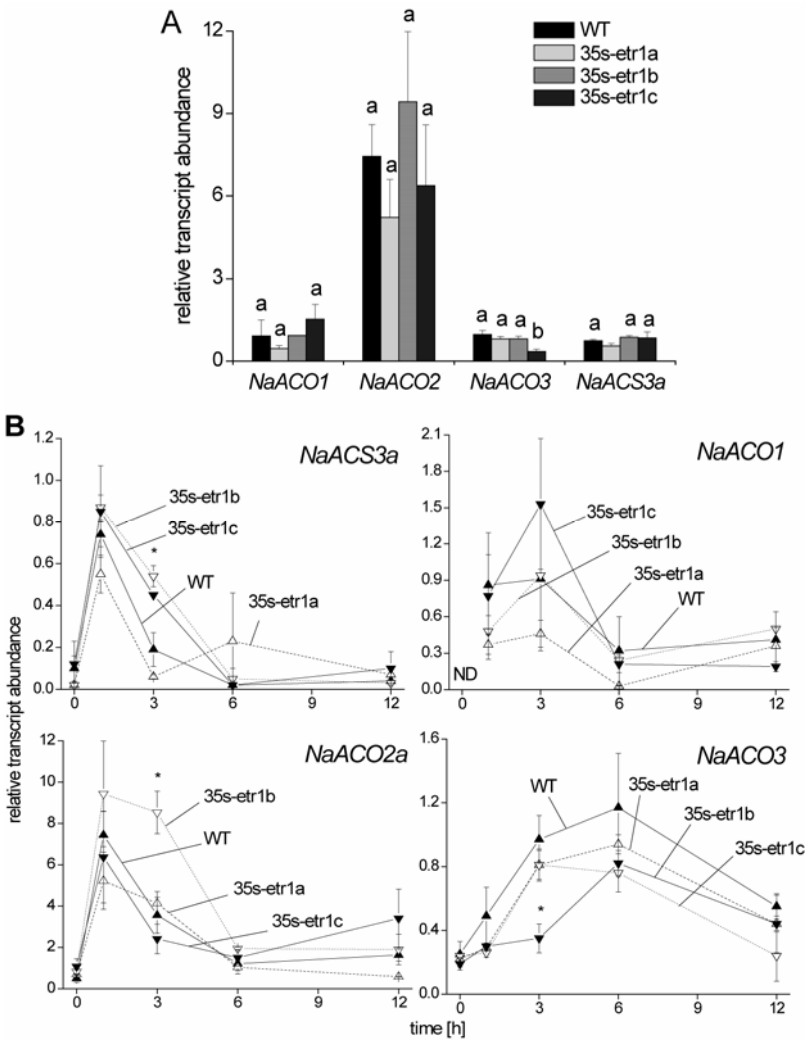

Figure S7. Transcript levels of ethylene biosynthesis genes are not altered in ethylene insensitive *N. attenuata* plants.

Mean relative transcript abundance ± SE (n = 3) of the indicated genes as analyzed by RT-PCR. **A.** Transcript abundance of *NaACS3a* and *NaACO2a* 1 h and of *NaACO1* and *NaACO3* 3 h after OS-elicitation in 35s-etr1a (light grey), 35s-etr1b (grey), 35s-etr1c (dark grey), and WT (black) plants. Transcript abundance was normalized to the endogenous control gene (ECI). Different letters indicate significance between the genotypes within the indicated gene ANOVA (Bonferroni-corrected *post-hoc* tests, $P < 0.05$). **B.** Transcript abundance ± SE of *NaACO1*, *NaACO2a*, *NaACO3*, and *NaACS3a* in 35s-etr1a (open up-triangles), 35s-etr1b (filled down-triangles), 35s-etr1c (open down-triangles), and WT (filled up-triangles) plants. Three individual plants were harvested at the indicated time points after elicitation by OS elicitation. Asterisks represent significant difference to WT plants at the respective time point ANOVA (Bonferroni-corrected post-hoc tests, $P < 0.05$).

10.3. Manuscript IV

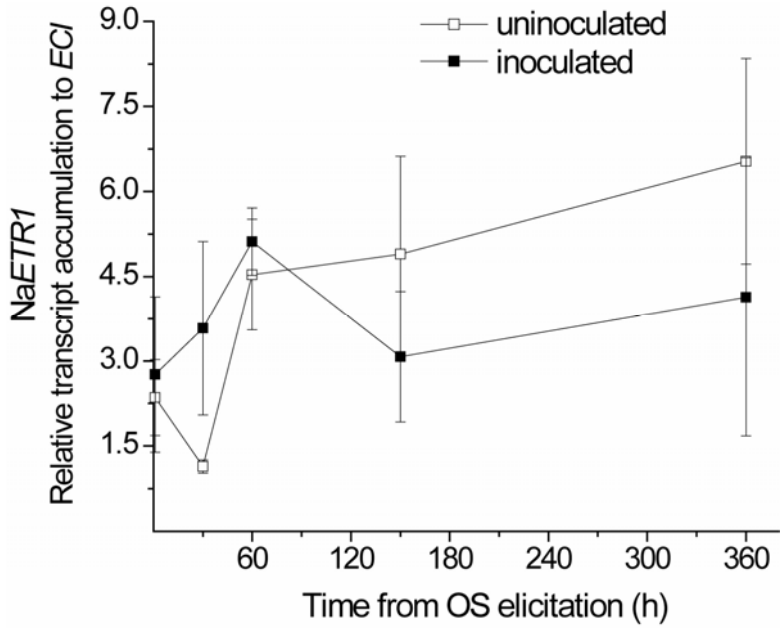

Figure S1. Transcript accumulation of ethylene receptor gene *NaETR1* in the leaves of OS-elicited uninoculated and *S. vermifera*-inoculated *N. attenuata*. Mean ± SE of the relative transcript levels in uninoculated (open symbols) and inoculated (filled symbols) WT plants, at the indicated time points following induction of the leaves by wounding and OS treatment. No significant differences were found between *S. vermifera*-inoculated and uninoculated plants at the indicated time points (*t*-test, $P > 0.05$)